Vol. 41, No. 3 May, 1954

THE JOURNAL
OF
BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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PUBLISHED MONTHLY
BY
THE JAPANESE BIOCHEMICAL SOCIETY
Tokyo University, Tokyo

AGENCIES

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Rutland, Vt.	Tokyo	Köln
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PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY, was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number, from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published bimonthly, one volume per year.

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editors*, *The Journal of Biochemistry*, *The Japanese Biochemical Society*, *c/o Departments of Biochemistry*, *Faculty of Medicine*, *Tokyo University*, *Bunkyo-ku*, *Tokyo*, *Japan.*

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The Journal of Biochemistry, Vol. 41, No. 3, 1954

STUDIES ON AEROBIC DECOMPOSITION OF CYSTEINE BY ESCHERICHIA COLI

II. EFFECTS OF VARIOUS RESPIRATORY SUBSTRATES AND INHIBITORS

By NOBUO TAMIYA

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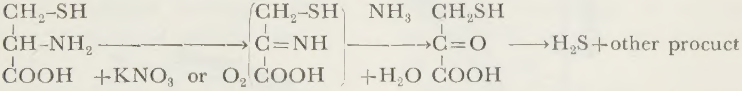
(Received for publication, November 27, 1953)

In the previous paper (*1*), it was reported that cysteine was decomposed by *E. coli* in the presence of oxygen or nitrate with the formation of hydrogen sulfide and ammonia as end products. It was also shown that the reaction was essentially different from that caused by cysteine desulfhydrase in two points, *i.e.*, (*1*) in its requirement for hydrogen acceptors such as oxygen or nitrate and (*2*) in the fact that the liberation of hydrogen sulfide was preceded by the splitting of ammonia from the substrate. From the available data in the previous paper, the author proposed the following stepwise reaction scheme:



$$\begin{matrix} CH_2\text{-}SH \\ \mid \\ CH\text{-}NH_2 \\ \mid \\ COOH \end{matrix} \xrightarrow[+KNO_3 \text{ or } O_2]{} \left(\begin{matrix} CH_2\text{-}SH \\ \mid \\ C{=}NH \\ \mid \\ COOH \end{matrix}\right) \xrightarrow[+H_2O]{NH_3} \begin{matrix} CH_2SH \\ \mid \\ C{=}O \\ \mid \\ COOH \end{matrix} \longrightarrow H_2S+\text{other procuct}$$

Characteristic of this reaction is also the fact that it is markedly stimulated by the coexistence of various respiratory substrates such as sugars or organic acids. The purpose of this paper is to present data dealing with the mechanism of the promoting action of these substances and the effect of various inhibitors on the reaction.

Experimental Procedure

The same methods as described in the previous paper were used throughout the experiments. Ammonia was measured by Conway's microdiffusion method (*2*) followed by Russell's (*3*) colorimetric determination. The hydrogen sulfide liberated was driven off from the reaction mixture by continuous aeration and estimated by St. Lorant's colorimetric method (*4*). Total amount of cysteine and cystine was determined by Mecham's method on the deproteinized reaction mixture (*5*). For the measurement of oxygen uptake, Warburg's manometric apparatus was used.

The Effect of Various Sugars and Organic Acids as Promotors

In the majority of experiments described in the previous paper, acetate or glucose was added as the "promoting substance." The same kind of promoting effect was exerted by a number of other substances which were investigated in the following experiments.

Pyruvic Acid—Pyruvic acid showed a remarkable promoting action on the aerobic desulfhydration of cysteine by *E. coli* as may be seen from Fig. 1; Curve A was obtained when sufficient amount of pyruvic acid was added, while Curve C represents the course of the reaction in the absence of the acid. When only an insufficient amount of pyruvic acid was added (Curve B), it was consumed at an intermediate stage of the reaction and the curve became parallel to C. At the point marked by the arrow in the figure, additional quantity of pyruvic acid was supplied, which caused a renewed increase in reaction rate as may be seen from the figure.

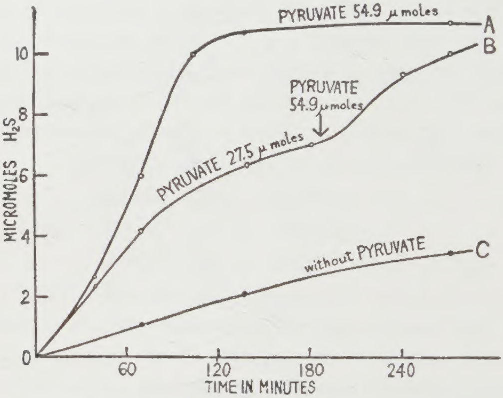


Fig. 1. Promoting effect of pyruvate on the cysteine decomposition by *E. coli*.

Contents of each tube: Cysteine hydrochloride—15.9 micromoles. Pyruvic acid—the amount indicated in the figure. Washed cells of *E. coli*—the amount in which 1.1 mg. of Kjeldahl's nitrogen was contained. *M*/15 phosphate buffer (pH 6.9) to 20 ml. Temperature: 37°. Gas phase: air.

The Ralation between the Amount of Promoting Substance and the Reaction Rate—In Fig. 2 are presented the experimental data with the addition of varying amount of acetate as promoting substance.

This experiment shows that the initial rate of the reaction was independent of the concentration of the promotor added, whereas the lower the initial concentration, the earlier the promoting effect disappeared.

Comparison of Promating Effects of Various Substances—The promoting effects of various substances were compared by adding them in sufficient quantities to the reaction mixture. Fig. 3 shows some of the results obtained in such experiments.

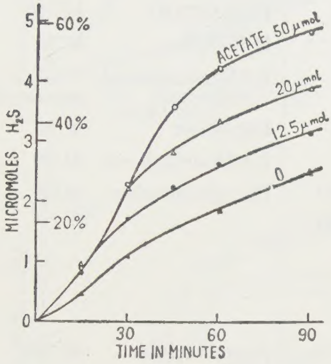


Fig. 2. Relation between the amount of promotor and the reaction rate.

Contents of each tube: Cystine—8.33 micromoles, Sodium acetate—the amount indicated in the figure. Washed cells of *E. coli*—the amount in which 1.1mg. of Kjeldahl's nitrogen was contained. *M*/15 phosphate buffer (pH 6.9) to 10ml. Temperature: 37°. Gas phase: air.

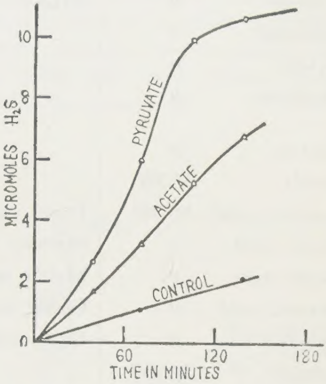


Fig. 3. Comparison of promoting effect of pyruvic acid and that of acetic acid.

Contents of each tube: Cysteine hydrochloride—15.9 micromoles. Promotor: Sodium acetate—100 micromoles or pyruvic acid—54. 9 micromoles. Washed cells of *E. coli*—the amount in which 1.12mg. of Kjeldahl's nitrogen was contained. *M*/15 phosphate buffer (pH 6.9) to 20ml.

Among the carbohydrates tested, which all displayed promoting action, glucose and saccharose proved to be most effective, showing their effect from the beginning of experiment. Galactose and arabinose, on the other hand, required an induction time of about 30 minutes before they exhibited the promoting activity. Lactose was found to be the least effective among the carbohydrates tested.

Organic acids such as succinic, lactic, pyruvic, acetic and fumaric acids, which are known to be metabolized rapidly by the bacteria, were

TABLE I

Effectors on the Aerobic Decomposition of Cysteine by E. coli

Promoting substances		Non effective substance		Inhibiting substances	
Saccharose	1 mg./10 ml.	NaF	*M*/240	KCN	*M*/10,000
Maltose	〃	$MgCl_2$	〃	H_3AsO_4	*M*/100
Glucose	〃	NH_4Cl	〃	H_3AsO_3	〃
Galactose	〃			FCH_2COOH	*M*/500
Xylose	〃			H_2N-NH_2	*M*/50
Arabinose	〃			2,4-Dinitrophenyl hydrazine,	saturated
Lactose	〃			Dimedone	*M*/50
Sorbit	*M*/200			2,4-Dinitrophenol	*M*/50
Succinic acid	*M*/200	Formic acid	*M*/370	Phenylhydrazine	*M*/50
Lactic acid	〃	Malonic acid	*M*/200		
Acetic acid	〃	Maleic acid	〃		
Pyruvic acid	〃	Oxalic acid	〃		
Fumaric acid	〃	Citric acid	〃		
Ethanol	*M*/200			Acetaldehyde	*M*/200
Glutamic acid	*M*/200	Glycine	*M*/200	Cysteic acid	*M*/200
Alanine	〃	Betaine	〃	Alanine	〃
Asparagine	〃			Leucine	〃
				Valine	〃
				Taurine	〃

all active as promotors, while some other kinds of acids, such as formic, maleic, oxalic, citric and malonic acids were found to be inactive. Malonic acid, which is a well known inhibitor for succinic oxidase, did not affect at all the liberation of hydrogen sulfide from cysteine irrespective of the presence or absence of succinic acid.

In Table I are summarized the comparative effects of the compounds tested.

The effect of amino acids and other substances will be dealt with in later sections.

In view of the fact that the compounds showing promoting effects are all common metabolic substrates or their intermediates, it may be inferred that in the absence of added promotors some endogenous substrates may be concerned in promoting the reaction.

The Reaction Step at which Promoting Substances Participate—As already

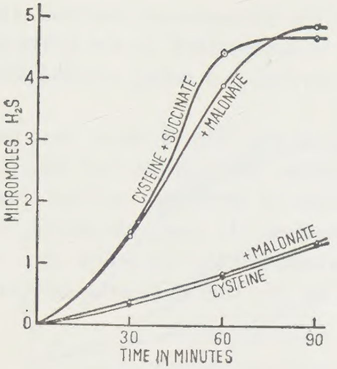


FIG. 4. Influence of malonate addition on the reaction rate which was promoted by succinate.

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Succinic acid (when added)—50 micromoles. Malonic acid (when added)—50 micromoles. Washed cells of *E. coli*—the amount in which 1.24 mg. of Kjeldahl's nitrogen was contained *M*/15 phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Cas phase: air.

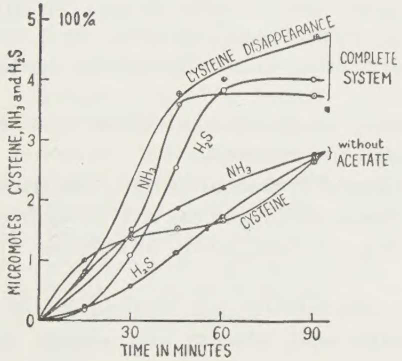


Fig. 5. Effects of acetate on the rates of disappearance of cysteine, production of ammonia and liberation of hydrogen sulfide.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Sodium acetate (when added)—25 micromoles. Dried cell preparation of *E. coli*—10 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Cas phase: air.

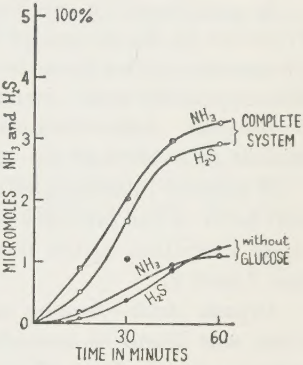


FIG. 6. Effects of glucose on the rates of production of ammonia and liberation of hydrogen sulfide.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Glucose—10 microles. Dried cell preparation of *E. coli* —10 mg. M/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Cas phase: air.

mentioned, the aerobic decomposition of cysteine by *E. coli* involves at least two reaction steps, of which one is the deamination of cysteine and the other is desulfhydrating breakdown of the deaminated intermediate. To make clear which of these two steps is accelerated by the effect of promotors, analytical experiments was carried out using acetate and glucose as promotors.

The experiments reproduced in Fig. 5 and Fig. 6 show that not only the decrease in total amount of cysteine and cystine but also the production of ammonia and the liberation of hydrogen sulfide were equally accelerated by the effect of promotors. It may, therefore, be inferred that the promoting substances accelerate the very first step of the reaction which may, most probably, be the step of deamination of cysteine.

Effects of Various Inhibitors

It was shown in the previous paper that the aerobic decomposition of cysteine by *E. coli* was inhibited by cyanide and arsenite. In a series of experiments, we have investigated other kinds of inhibitors for their effects upon the same reaction.

Inorganic Salts—Sodium fluoride, magnesium chloride and ammonium chloride had no effect upon the reaction.

A marked inhibitory effect was found in arsenate which, like arsenite dealt with in the previous paper, affects preferably the step of hydrogen sulfide liberation rather than the step of deamination of cysteine. (see Figs. 7 and 8)

Organic Acids—Among organic acids investigated, marked inhibitory action was found in monofluoroacetic acid, alanine and cysteic acid, while taurine had no effect.

The effect of alanine will be discussed in detail in the next section.

Hydrazine and Its Derivatives—Fromageot (*6*) reported the inhibitory effect of hydrazine and other reagents for ketonic group upon the action of cysteine desulfhydrase. As may be seen from Fig. 9, hydrazine, phenylhydrazine, 2,4-dinitrophenylhydrazine and dimedone inhibited the hydrogen sulfide liberation also under aerobic condition.

Phenylhydrazine did not affect at all the respiration of the bacteria with glucose as substrate at the concentration used in this experiment (see Fig. 20), but it completely halted the ammonia production from alanine at the same concentration. (see Fig. 21). As will be shown later, the production of ammonia from alanine seems to be catalyzed by the same enzyme system as the deamination of cysteine. The reaction step

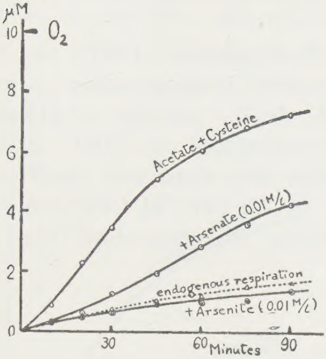


FIG. 7. Inhibiting effect of arsenate or arsenite on the respiration of *E. coli* with acetate and cysteine as substrates.

Contents of each Warburg's vessel: Cysteine hydrochloride—1 micromole. Sodium acetate—5 micromoles. Inhibitor—10 micromoles. Inhibitor—10 micromoles. Dried preparation of *E. coli*—2mg. *M*/15 phosphate buffer (pH 6.9) to 1 ml. 0.2 ml. of 5% KOH in the center well. Temperature: 37°. Gas phase: air.

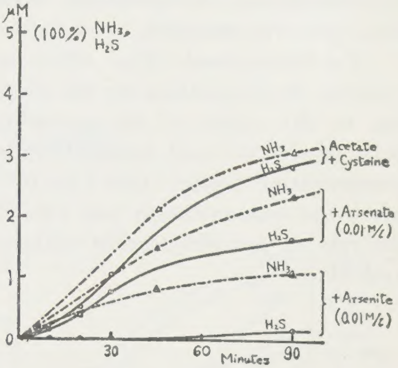
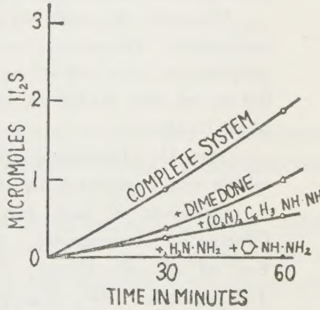


FIG. 8. Inhibiting effect of arsenite or arsenate on the hydrogen sulfide liberation from cysteine by *E. coli*.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Sodium acetate—25 micromoles. Inhibitor—50 micromoles. Dried preparation of *E. coli*—10 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

FIG. 9. Inhibiting action of dimedone and hydrazines on the hydrogen sulfide liberation from cysteine by *E. coli*.

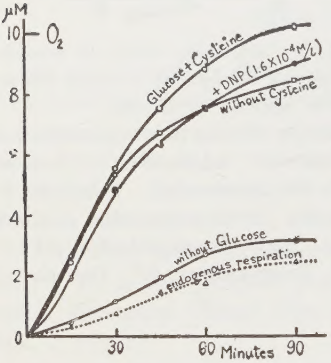
Contents of each tube: Cysteine hydrochloride—5.35 micromoles. Sodium acetate—100 micromoles. Inhibitor—100 micromoles Washed cells of *E. coli*.—the amount in which 1.0 mg. of Kjeldahl's nitrogen was contained, *M*/15 phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.



attacked by phenylhydrazine may, therefore, most probably be the deamination of cysteine, which represents the first step in the decomposition of cysteine by *E. coli* under aerobic condition.

Acetaldehyde—Acetaldehyde exhibited a fairly strong inhibitory action upon the reaction.

2,4-Dinitrophenol—The effect of 2,4-dinitrophenol (DNP) on the cysteine decomposition by the bacterium varied to some extent according to the nature of the promotor used. In the presence of glucose, both respiration and desulfhydration were inhibited by DNP at the concentration higher than $1.6\times10^{-3}\,M$ while they remained unaffected when the concentration was $1.6\times10^{-5}\,M$. At $1.6\times10^{-4}\,M$, DNP reduced the rate of desulfhydration without affecting the respiration (Figs. 10 and 11).



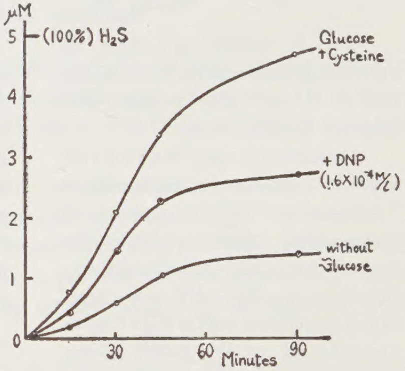


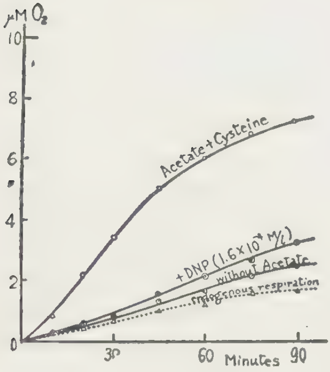
FIG. 10. Effect of DNP on the respiration of the cell preparation with glucose and cysteine as substrates.

Contents of each Warburg's vessel: Cysteine hydrochloride—1 micromole. Glucose—2 micromoles. DNP—0.16 micromoles. Dried preparation of *E. coli*—2 mg. *M*/15 phosphate buffer to 1 ml. (pH 6.9). 0.2 ml. of 5% KOH in the center well. Temperature: 37°. Gas phase: air.

FIG. 11. Inhibiting action of DNP on the cysteine decomposition which was stimulated by the addition of glucose.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Glucose—10 micromoles. DNP—0.8 micromoles, Dried cell preparation of *E. coli*—10 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Cas phase: air.

Qualitatively similar, but appreciably stronger effect of DNP was observed in the presence of acetate. When DNP was added at the concentration of $1.6 \times 10^{-4}\,M$ the rate of desulfhydration was reduced almost to the level observed in the absence of promotors.



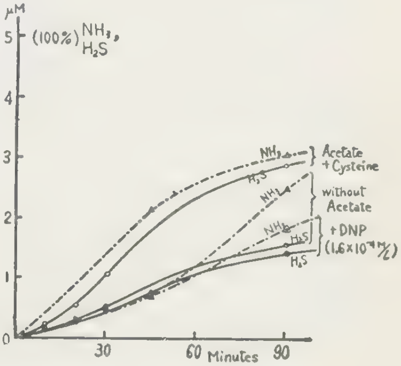


FIG. 12. Effect of DNP on the respiration of the cell preparation with acetate and cysteine as substrates.

Contents of each Warburg's vessel: Cysteine hydrochloride—1 micromole. Sodium acetate—5 micromoles. DNP—0.16 micromoles. Dried preparation of *E. coli*—2 mg. (side arm). *M*/15 phosphate buffer (pH 6.9) to 1 ml., 0.2 ml. of 5% KOH in the center well. Temperature: 37°. Gas phase: air.

FIG. 13. Inhibiting action of DNP on the aerobic decomposition of cysteine by *E. coli*.

Contents of each tube: Cysteine hydrochloride—5 micromoles. Sodium acetate—25 micromoles. DNP—0.8 micromoles. Dried preparation of *E. coli*—10 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

Strong inhibition of respiration with acetate as substrate was caused by DNP at the concentration of $1.6 \times 10^{-4}\,M$, at which no inhibition was observed when glucose was used as substrate (Fig. 10).

Based on the fact that when glucose was added as promotor, DNP exerts, at considerably low concentrations, inhibitory action on the liberation of hydrogen sulfide without affecting the respiration, one might surmise that the process of cysteine decomposition involve, or closely. related to, the phosphorylation reaction which is known to be specifically susceptible to the action of the inhibitor.

Suppression of the respiration with acetate by DNP seems to indicate the complexity of the acetate oxidizing mechanism compared with that of glucose.

Relation between the Aerobic Decomposition of Cysteine and That of Other Amino Acids

If, as has been inferred in the previous paper, the decomposition of cysteine by *E. coli* involves the step of oxidative deamination of cysteine, there may be the possibility that the enzyme system concerned will act in a similar manner also on some other amino acids and that, if this is the case, the deamination of cysteine may be interfered with by the existence of these amino acids. From this point of view, the behavior of bacterial cells towards some amino acids was investigated.

Effect of Alanine—As shown in Fig. 10, alanine, when added alone to the reaction mixture, considerably promoted the liberation of hydrogen sulfide from cysteine. On the contrary, when it was added together with other promoting substance such as acetate, it inhibited the reaction.

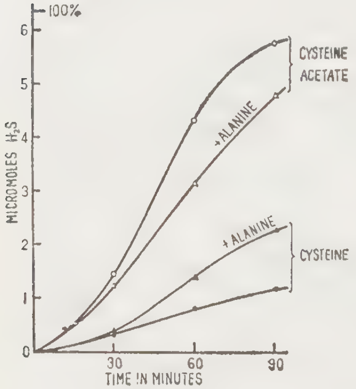


FIG. 14. Effect of alanine on the aerobic decomposition of cysteine.

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Sodium acetate—150 micromoles. D, L-alanine—50 micromoles. Washed cells of *E. coli*—the amount in which 1.05 mg. of Kjeldahl's nitrogen was contained. *M*/15 phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.

It may be supposed that alanine, when added alone, behaved as a promoting substance by virtue of its deaminated product (pyruvic acid), but that, in the presence of other suitable promotors, it competed with cysteine in being deaminated by the bacteria.

To test the validity of this assumption, the deamination of alanine by the bacteria was investigated more in detail in comparison with the corresponding reaction of cysteine. As it was the case with cysteine, the production of ammonia from alanine by *E. coli* under aerobic con-

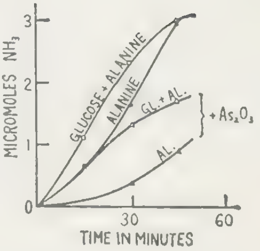


Fig. 15. Promoting effect of glucose on the decompostion of alanine by *E. coli.*

Contents of each tube: D, L-Alanine—5 micromoles. Glucose—20 micromoles. Arsenic acid—50 micromoles. Dried preparation of *E. coli*—5 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

ditions was stimulated by the addition of glucose. This phenomenon was observed more clearly when arsenite was added to the medium. (Fig. 15)

Another similarity between the deamination of cysteine and that of alanine was found in the effect to 2, 4-dinitrophenol, which, at the concentration low enough to exhibit no effect on the respiration of bacteria with glucose as substrate, markedly suppressed the production of ammonia both from alanine and from cysteine. (see also Figs. 10 and 11)

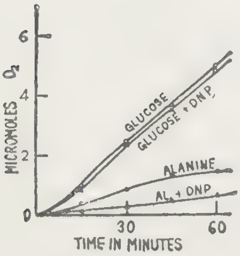


Fig. 16. Effect of DNP on the respiration of *E. coli* with glucose and alanine as substrates.

Contents of each Warburg vessel: D, L-Alanine—1 micromole. Glucose—4 micromoles. DNP—0.16 micromoles. Dried preparation of *E. coli*—1 mg. *M*/15 phosphate buffer (pH 6.9) to 1 ml. 0.2 ml. of 5% KOH in the center well. Temperature: 37°C. Gas phase: air.

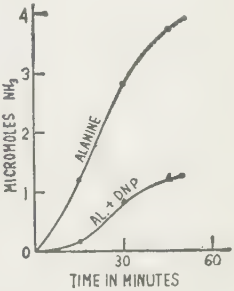


Fig. 17. Effect of DNP on the ammonia liberation from alanine by *E. coli*

Contents of each tube: D, L-Alanine—5 micromoles. Glucose—20 micromoles. DNP—0.8 micromoles. Dried preparation of *E. coli*—5 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

Parallelism between these two processes was also observed in the effect of cyanide, which inhibited almost completely the deamination as well as the respiration of alanine and glucose as substrates, as it was the case in cysteine decomposition.

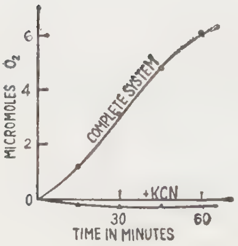


FIG. 18. Inhibition of bacterial respiration by cyanide with glucose and alanine as substrates.

Contents of each Warburg's vessel: D, L-Alanine—1 micromole. Glucose—4 micromoles. Potassium cyanide—2 micromoles. Dried preparation of *E. coli*—1 mg. *M*/15 phosphate buffer (pH 6.9) to 1 ml. 0.2 ml. of 5% KOH in the center well. Temperature: 37°. Gas phase: air.

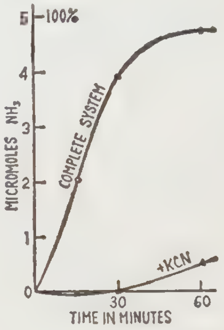


FIG. 19. Inhibiting effect of cyanide on the liberation of ammonia from alanine.

Contents of each tube: D, L-Alanine—5 micromoles. Glucose—20 micromoles. Potassium cyanide—10 micromoles. Dried preparation of *E. coli*—5 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

Furthermore, it was found that the deamination of alanine was completely ceased by 10^{-2} *M* phenylhydrazine, by which the respiration was not affected at all.

These observations make it appear highly probable that the deamination of alanine and that of cysteine are caused by a common enzyme system.

Effects of Other Amino Acids—L-Leucine, L-valine, glycine and betaine were found to affect only slightly or not at all the decomposition of cysteine irrespective of the presence or absence of acetate. In our experimental conditions, these substances themselves were not attacked at all by the bacterium.

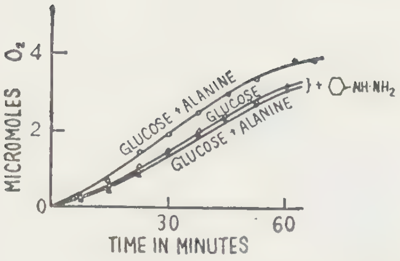


Fig. 20. Effect of phenylhydrazine on the respiration of *E. coli* with glucose and alanine as substrates.

Contents of each Warburg's vessel: D,L-Alanine—1 micromole. Glucose—4 micromoles. Phenylhydrazine—10 micromoles. Dried preparation of *E. coli* —1 mg. *M*/15 phosphate buffer (pH 6.9) to 1 ml. 0.2 ml. of 5% KOH in the center well. Temperature: 37°. Gas phase: air.

(The values in the figure are not corrected for endogenous respiration.)

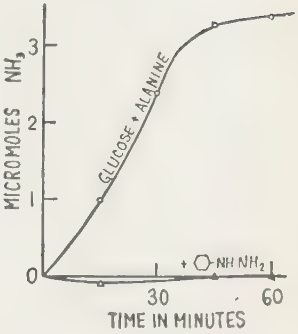


Fig. 21. Inhibiting effect of phenylhydrazine on the ammonia liberation from alanine by *E. coli*.

Contents of each tube: D,L-Alanine—5 micromoles, Glucose—20 micromoles. Pheylhydrazine—50 micromole. Dried preparation of *E. coli*—5 mg. *M*/15 phosphate buffer (pH 6.9) to 5 ml. Temperature: 37°. Gas phase: air.

L-Asparagine and L-glutamic acid exhibited strong promoting effects upon the liberation of hydrogen sulfide from cysteine both in the presence and absence of other promoting substances. These effects may be due to the existence of the easily oxidizable carbon residue in these amino acids.

DISCUSSION

Based on the data described in the previous and present papers, the course of events occuring in the aerobic decomposition of cysteine by *E. coli* may be represented as follows:

Reaction (1) requires the presence of oxygen or nitrate and is inhibited by alanine, hydrazines and dimedone. This step of the reaction is stimulated by the addition of various respiratory or fermentative substrates such as acetate or glucose. The metabolic breakdown of these

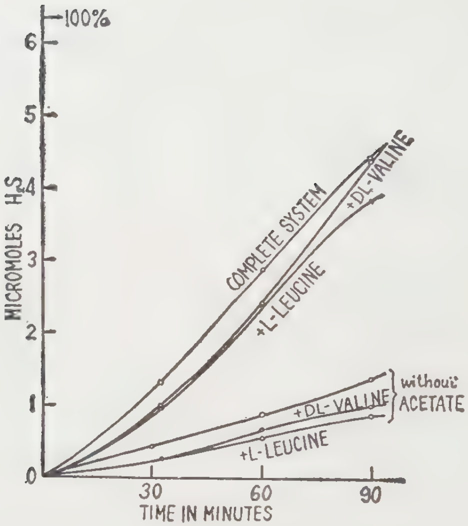


FIG. 22. Effects of L-leucine and of D, L-valine on the aerobic decomposition of cysteine by *E. coli*.

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Sodium acetate—50 micromoles. Amino acid—50 micromoles. Washed cells of *E. coli*—the amount in which 0.872 mg. of Kjeldahl's nitrogen was contained. *M*/15 phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.

promoting substances are inhibited by potassium cyanide or monofluoroacetate. This may be the reason why the cysteine decomposition is suppressed by the effect of these substances. The mechanism, by which

$$\begin{array}{ll}
\mathrm{HS{-}CH_2{-}CH(NH_2){-}COOH} & \\
\quad\downarrow & (1) \\
\mathrm{[HS{-}CH_2{-}C(=NH){-}COOH]} & \\
\quad\downarrow & (2) \\
\mathrm{NH_3+HS{-}CH_2{-}CO{-}COOH} & \\
\quad\downarrow & (3) \\
\mathrm{H_2S+other\ products} &
\end{array}$$

the promoting substances exert the stimulating activity on the reaction (1) is not yet clear, but it is probable that some phosphorylation reaction is involved in it, because the promoting action is inhibited strongly by the addition of small amount of DNP. Similar promoting effect of respiratory process upon decomposition of amino acids by micro-

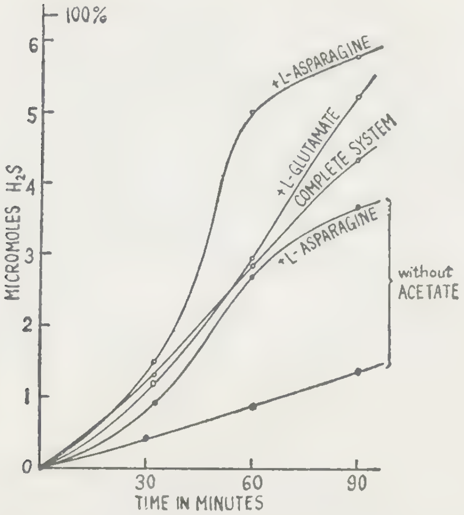


FIG. 23. Effects of L-asparagine and of L-glutamic acid on the aerobic decomposition of cysteine by *E. coli.*

Contents of each tube: Cysteine hydrochloride—6.35 micromoles. Sodium acetate—50 micromoles. Amino acid—50 micromoles. Washed cells of *E. coli*—the amount in which 0.872 mg. of Kjeldahl's nitrogen was contained. *M*/15 phosphate buffer (pH 6.9) to 10 ml. Temperature: 37°. Gas phase: air.

organism has been suggested by Geiger (*7*). The promotion of this kind is reported by Umbreit (*8*) to be supressed by the effect of streptomycine.

Reaction (*2*) is supposed to proceed spontaneously.

As to reaction (3), it is inhibited by arsenite and arsenate more strongly than reaction (1). It was by taking advantage of this property of arsenite that we have succeeded in obtaining the intermediate product, thiol pyruvic acid, from the reaction mixture, as reported in the previous paper.

The author is very grateful to Prof. Soda for his continuing guidance through the course of this research. The author also wish to thank Drs. Y. Kiho, Y. Kondo and J. Niwa for their co-operation and many useful discussions.

SUMMARY

1. The aerobic decomposition of cysteine by *E. coli* is accelerated

by the effect of various sugars and organic acids. This effect seems to be due to the acceleration of deamination of cysteine, which represents the first step in the decomposition of the substrate.

2. The promcting effect shown by these compounds is inhibited by 2, 4-dinitrophenol, indicating that the step of deamination may involve some phosphorylation process.

3. The step of deamination of cysteine is affected also by the effect of some amino acids, and especially notably by alanine which seems to be dealt with by the same enzyme system concerned in the deamination of cysteine.

4. Effects of several kinds of inhibitors were studied.

5. The reaction mechanism of the aerobic breakdown of cysteine by *E. coli* was discussed.

REFERENCES

(*1*) Tamiya, N., *J. Biochem.*, **41**, 199 (1954)
(*2*) Conway, E. J., Microdiffusion analysis and volumetric error, Crosby Lockwook and Son Ltd. London, (1950)
(*3*) Russell, J. A., *J. Biol. Chem.*, **156**, 457 (1945)
(*4*) Freudenberg, K., and Münch, A., *Z. physiol. Chem.*, **263**, 13 (1940)
(*5*) Mecham, D. K., *J. Biol. Chem.*, **151**, 643 (1943)
(*6*) Fromageot, C., and Tchen Pau Kiun, *Bull. Soc. Chim. biol.*, **25**, 1141 (1943)
(*7*) Geiger, W. D., *Arch. Biochem.*, **15**, 227 (1947)
(*8*) Umbreit, W. W., *J. Biol. Chem.*, **177**, 703 (1949)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

BILE ACIDS AND STEROIDS

II. STUDIES ON HOG BILE ACIDS (PART 2). PROGESTERONE FROM HYODESOXYCHOLIC ACID

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(Received for publication, November 27, 1953)

In the previous paper (*1*), we have described on the partial oxidation of α-hyodesoxycholic acid (*2*, *3*, *4*) with *N*-bromosuccinimide, by which we have obtained 3-keto-6-hydroxycholanic acid in good yield.

In the present investigation, pregnane-3,6-diol-20-one (*5*), which was obtained by degradation of side chain of α-hyodesoxycholic acid, has been made trial oxidation with NBS.

We were thus able to confirm that pregnane-3,6-diol-20-one (I) was partially oxidized at C_3-hydroxy group in the same manner. By the use of 1.2 equivalents NBS, *ca.* 30 per cent of starting material was recovered.

But when the excess of NBS (2 equivalents) was used, some allopregnane-3,6,20-trione (III) (1∼2.5 per cent) was obtained as a by-product.

Oxidation product so obtained showed no tendency of crystallization. Attempt to crystallize this oily oxidation product was unsuccessful. Zimmermann and Jaffé color tests were positive. However, the structure of this oily product was assumed to be pregnane-6-ol-3, 20-dion (II) with the following results: The infrared spectra* of this oxidation product have absorption bands in the region near 2.90 and 5.88 μ, attributable to the streching vibration of O-H and C=O bonds respectively. This structure was also presumed by comparing of paper partition chromatograms by K. Savard's method (*6*) (Fig. 1).

Then, by the dehydration with phosphorus oxychloride in pyridine, the oxidation product (II) affords progesterone with the migration of one double bond.

It is very interesting that in the partial oxidation of the pregnane nucleus, allo-pregnan-3,6,20-trione (III) was obtained even in poor yield. But, whether it was allomerized in the course of reaction or of purification by chromatography on alumina, was not further investigated.

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* The infrared absorption curves were recorded on a Pekin-Elmer Model 12-C spectrometer with automatic slit drive in Nujol mull or film.

The author's thanks are tendered to Mr. E. Hirai, Mr. K. Tori and Mr. Y. Matsui of this laboratory for the microanalyses and for the photographing of ultraviolet- and infared-spectra.

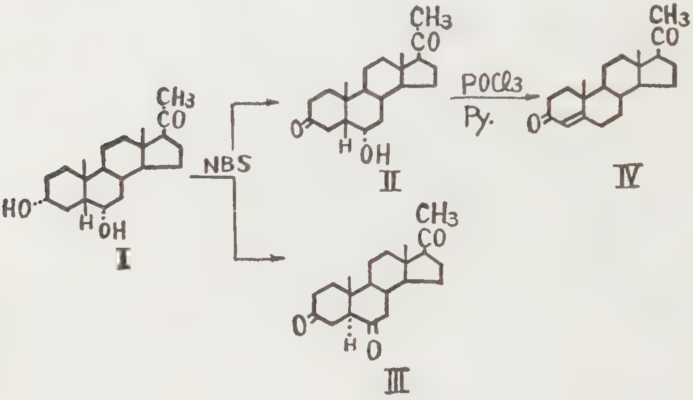


Chart 1

EXPERIMENTAL

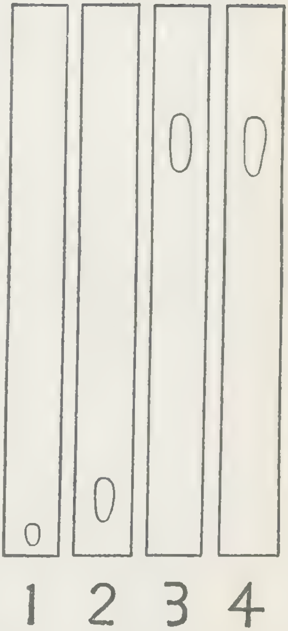
All melting points are not corrected.

(*1*) *Pregnane*-6-*hydroxy*-3,20-*dione* (*II*)—0.6 g. of pregnane 3α, 6α diol-20-one (I) (prepared from α-hyodesoxycholic acid according to Hoehn (*5*)) was dissolved in 15 ml. of acetone and 1.5 ml. of water, 0.5g. (*ca.* 2 equivalents) of NBS was then added, and the reaction mixture was allowed to stand overnight at room temperature (27°).

The solvent was removed by distillation under diminshed pressure and the residue was extracted with ether. The organic solvent layer was washed consecutively with water, dilute alkaline and again water. After drying over with sodium sulfate, the residue was purified by chromatography on alumina (8 g.). First elutions with benzene (*ca.* 200 ml.) gave *ca.* 50 mg. of oily product which caused crystallization by treatment with ether-petroleum-ether.

Further elution with benzene (*ca.* 1,200 ml.) gave *ca.* 0.6 g. of the principal oxidation product. Attempts to crystallize this oily oxidation product were unsuccessful. But this structure was presumed as pregnane-6-hydroxy-3,20-dione by color tests, by infrared spectra* and by comparing of paper partition chromatograms of some pregnane derivatives.

1. Pregnane-3,6-diol-20-one. (**I**)
2. Pregnane-6-hydroxy-3,20-dione. (**II**)
3. Pregnane-3,6,20-trione.
4. Allo-pregnane-3,6,20-trione. (**III**)



The substance from first elution melted at 205–207°. Yield, 15 mg. Recrystallized from 94 per cent ethylalcohol, the compound forms the clusters of prisms of melting point, 212–215°. The infrared spectra* showed no free hydroxyl group but absorption band at 5.87 μ. The spectra of pregnane-3,6,20-trione have absorption bands at 5.86 and 5.88 μ. It affords violet color for the Zimmermann test and orange-yellow color for the Jaffé test. It showed no depression on admixture with authentic allopregnane-3,6,20-trione.

Analytical values:

Calcd. for $C_{21}H_{30}O_3$, C, 76.32%; H, 9.15%
Found, C, 76.21%; H, 9.14%

When 1.2 equivalents of NBS were used as the oxidation reagent, ca. 30 per cent of starting material was separated by chromatography on alumina.

(2) *Allomerization of Pregnane-3,6,20--trione by Chromatographying on Alumina*—*ca*. 50mg. of authentic pregnane-3,6,20-trione (m.p., 183–184°) was subjected to Al_2O_3 (0.5 g.) chromatography and was eluted by benzene. The substance thus obtained melted at 214–217°. $[\alpha]_D^{21}$ +42.7° (in dioxane, C=0.475, l=0.5 dm.). It showed no depression on admixture with authentic allo-pregnane-3,6,20-trione.

(3) *Progesterone* (*IV*)—0.6 g. of oily oxidation product described above was dissolved in pyridine (15 ml.). Approximately 4 ml. of freshly redistilled phosphorus oxychloride was added and the mixture was allowed to stand overnight at room temperature (31°). After decomposition with dilute hydrochloric acid solution and ice-water, the dehydration product was extracted with benzene. The benzene layer was washed consecutively with dilute acid, dilute alkaline and again with water.

The neutral benzene solution was extracted with 50 per cent sulfuric acid and the acidic extracts were diluted with twice volumes of water. The diluted acidic solution was re-extracted with ether. After washing with water and drying over with sodium sulfate, the solvent was removed. The oily product was crystallized by means of acetone-water or by chromatographic separation on alumina (8 g.).

Recrystallized from ether-*n*-hexan or ether-petroleum-ether, it gave the needle crystall of melting point, 117–119°. Yield, *ca*. 50 mg. Recrystallized from acetone-water, it melted at 125–127°.

The compound showed no depression of the melting point on admixture with an authentic progesterone (Syntex Co.). The absorption spectrum of this compound showed a maximum at 241 mμ (log. E=4.24). $[\alpha]_D^{20} + 191°$ (in ethyl alcohol, C=0.457 l=1 dm.).

Analytical values:

Cald. for $C_{21}H_{30}O_2$,	C, 80.2%;	H, 7.6%
Found,	C, 80.51%;	H, 7.70%

SUMMARY

In the previous paper, it was reported that methyl α-hyodesoxycholate was partially oxidized only at C_3-hydroxy group with NBS. By the same oxidation procedure pregnane-3(α)-6(α)-diol-20-one, derived from α-hyodesoxycholate, afforded pregnan-6(α)-hydroxy-3,20-dione, which was dehydrated to progresterone. By using excess *N*-bromosuccinimide, it afforded some allo-pregnane-3,6,20-trione as a by-product.

REFERENCE

(*1*) Takeda, K., and Kawanami, J., *J. Biochem.* **40**, 477 (1953)
(*2*) Marker, R.E., and Krüger, J., *J. Am. Chem. Soc.*, **62**, 72 (1940)
(*3*) Yamazaki, K., private communication.
(*4*) Gallagher, T.F., and Xenos, J.R., *J. Biol. Chem.*, **165**, 365 (1946)
(*5*) Moffett, R.B., Stafford, J.E., and Hoehn, W.E., *J. Am. Chem. Soc.*, **68**, 1857 (1946)
(*6*) Savard, K., *J. Biol. Chem.*, **202**, 457 (1953)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

ADAPTIVE UTILIZATION OF SEDOHEPTULOSE BY BACTERIA*

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(Received for publication, December 3, 1953)

Recent establishement of sedoheptulose phosphate as one of the early products in photosynthesis (*1–3*) has greatly stimulated interest in the metabolic significance of this seven-carbon sugar, originally discovered in the *Sedum* plant (*4*). The phosphate ester of this sugar has also been identified as a product of pentose and hexose phosphates metabolism with enzymes of animal origin (*5*, *6*). Further, an enzyme preparation from peas has been reported to catalyse the reaction of D-erythrose with triose phosphate to give sedoheptulose phosphate (*7*). The hexosemonophosphate fraction isolated from yeast also has been found to contain a small amount of a ketoheptose ester (*8*). Despite these findings suggesting some important functions of sedoheptulose in general metabolism, little information is available as yet concerning the metabolic behaviour of bacteria towards the heptose.

In an attempt to ascertain the possible role of sedoheptulose in aromatic biosynthesis suggested by several authors (*9–11*), we have found that the heptose is rapidly metabolized by certain bacteria previously adapted to this sugar. Further study of this utilization process has provided evidence that the initial step of the heptose metabolism may be the phosphorylation of the sugar by ATP.** An enzyme formed in the adapted cells catalyzes the reaction,

$$\text{Sedoheptulose}+\text{ATP}\longrightarrow\text{sedoheptulose phosphate}+\text{ADP}.$$

MATERIALS AND METHODS

Bacterial Strains—Three strains of bacteria capable of utilizing sedoheptulose were

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[*] Presented at the 25th Annual Meeting of the Japanese Biochemical Society, April, 1953, Tokyo. An abstract appeared in Japanese in *J. Jap. Biochem. Soc.*, **25**, 55 (1953).

[**] ATP=Adenosine triphosphate; ADP=Adenosine diphosphate.

isolated through enrichment culture; they were designated as W-1, W-2, and Y-1, respectively. Since preliminary experiments with these three strains showed that the strain W-2 possessed the most powerful activity, subsequent studies were conducted only with this strain. Bacteriological tests indicated that this strain is a Gram-positive and spore-forming rod, probably belonging to the genus *Bacillus.*

Cell Suspension—Most experiments were carried out with washed suspensions of the sedoheptulose-adapted cells prepared as follows: The strain W-2 was cultivated for 20-22 hours at 35° on a peptone-broth-agar medium containing 0.1 per cent sedoheptulose (pH 7.2). The cells harvested from the medium were washed three times with 0.9 per cent saline solution and suspended in distilled water (10-30 mg. of dry weight per ml.). In some of the experiments acetone powder of the adapted cells (prepared according to the direction of Umbreit (*12*)) was used instead of the living cells. The powder was suspended in distilled water (10-30 mg. per ml.).

Sedoheptulose—Sedoheptulose was isolated as purified syrup from the succulent plant *Sedum japonicum* according to the procedure of Nordal (*13*). The yield of the syrup was about 0.4 per cent of the fresh material. The purity of the syrup was estimated to be 93 per cent on the basis of the yield of the crystalline derivative phenylosazone (*13*). With a paper chromatographic method using ethyl acetate-acetic acid-water (3 : 1 : 3) as solvent no impurities of carbohydrate nature could be detected in the syrup. Aniline hydrogen phthalate (*14*) and orcinol-trichloroacetic acid (*15*, *16*) were used for detection.

ATP—Barium salt of ATP was prepared by the method of Szent-Györgyi (*17*). The prity of the preparation prepared by this method is stated to be about 90 per cent (*17*). The preparation was converted to sodium salt before use.

Growth Experiment—The conditions for the growth experiments were as follows; the bacterium was cultivated at 35° in 50 ml. of a liquid medium (referred to as *Medium S*). The composition of this medium was as follows; sedoheptulose, 0.2; peptone, 0.1; K_2HPO_4, 0.7; KH_2PO_4, 0.3; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.01; H_2O, 100; pH 6.8. At intervals aliquotes were taken from the medium and used both for growth measurement and for sedoheptulose determination. The growth was measured by determining the optical density of the medium with the use of a Hitachi Model "A" photoelectric colorimeter ("G" filter).

Utillization by Cell Suspension—The reaction mixture was as follows; 0.5 ml. of the cell suspension, 0.5 ml. of $M/5$ phosphate or veronal buffer, and 0.2 ml. of 10^{-2} M sedoheptulose in a total volume of 2.0 ml. When necessary, various additions were made to this mixture keeping the total volume always at 2.0 ml. Except for the experiments on the effect of pH, the pH was fixed to 6.0. The reaction mixture was shaken in a small reagent bottle stoppered with cotton at 35°. In the anaerobic experiments Thumberg tubes were used instead of the cotton-plugged bottles. After desired periods of shaking the reaction was stopped by deproteinizing with a mixture of 0.5 ml. of 20 per cent mercuric acetate and 0.5 ml. of 10 per cent sodium acetate. The utilization of sedoheptulose was followed by determining the amount of the heptose in the deproteinized filtrate.

Determination of Sedoheptulose—The determination of sedoheptulose was carried out by the method of Nordal and Klevstrand (*18*) with the following modifications. After deproteinizing the reaction mixture with mercuric acetate and sodium acetate as above, 1 ml. of the filtrate was taken into a test tube. After development of a blue colour with 1 ml. of Bial reagent (50 mg. of orcinol was dissolved in 25 ml. of concentrated hydrochloric acid) and by heating, the colour was measured by the photoelectric colorimeter using "R" filter. It was found that the selection of deproteinizing reagent was particularly important for the accuracy of the determination. Acidic reagents such as trichloroacetic acid or perchloric acid as well as alkaline ones such as zinc or cadmium hydroxides were all inadequate for the purpose. This is probably due to extraction of Bial-sensitive substances from the cells. Using a mixture of mercuric and sodium acetates for deproteinization, no colour was developed in cells containing no heptose.

RESULTS

Utilization of Sedoheptulose during Growth—When the strain W-2 was cultivated in *Medium S*, a considerable growth was observed after 24 hours incubation. At the same time it was found that the sedoheptulose originally present in the medium was almost completely exhausted during this period. Subsequently, the disappearance of the heptose was followed in relation to the growth of the strain W-2. Typical results of these experiments are illustrated in Fig. 1.

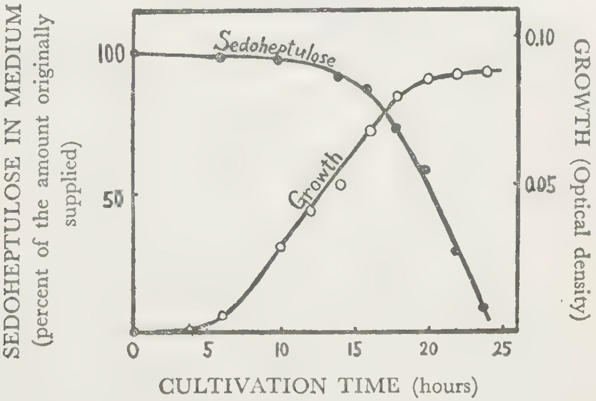

FIG. 1. Utilization of sedoheptulose by the strain W-2 in relation to growth.

As may be seen from these curves, a rapid utilization of the sugar takes place only after the growth approaches the stationary phase. Just a small decrease of heptose was observed after 16 hours of cultivation, although the growth curve had reached about three quarters of the maximum height after this period of time. This fact strongly suggested that the cells must be adapted to the sugar prior to the rapid utilization.

It was also noted that the pH of the medium did not change appreciably throughout the course of cultivation, suggesting that no large-scale production of acids occurred in the process.

A strain of *Escherichia coli* was also able to grow in the same medium, but the growth was less than that of strain W-2, and the sugar was utilized only to a very small extent.

Adaptive Nature of Sedoheptulose-utilizing System—The adaptation of the cells for utilizing the heptose was clearly shown by experiments in which washed cells of the organism grown in media containing various sugars were shaken with sedoheptulose. As shown in Table I, it was confirmed that the cells grown in the presence of sedoheptulose are capable of consuming actively the heptose under the shaking conditions. The cells grown on the other sugars, however, were nearly or completely devoid of the enzyme system responsible for the utilization of heptose. The slight effect of fructose and sucrose to stimulate the formation of the heptose enzyme system might be of some interest, since these sugars are similar to sedoheptulose in having a ketose structure. It will be noted that glucose has no such effect.

TABLE I

Sedoheptulose-utilizing Activity of the Cells Grown on Various Sugars

Sugars in medium	Sedoheptulose consumed	Dry weight of cells	Sedoheptulose consumed per mg. of dry weight
Sedoheptulose	438 $\mu g.$	16.4 *mg.*	27 $\mu g.$
Glucose	0	13.0	0
Fructose	53	23.8	2
Sucrose	84	20.5	4
None	5	15.2	0.3

The strain W-2 was grown in medium S each containing 0.2 per cent of the sugar indicated instead of sedoheptulose of the original medium. Washed suspension from each medium was shaken at 35° for 90 minutes in *M*/20 phosphate buffer (pH 6.0) with 560 μg. of sedoheptulose.

Effect of pH—The effect of pH on the utilization process was studied with washed cells of the sedoheptulose-adapted organism. As shown in Fig. 2, the pH-activity curve has two peaks at about pH 6 and pH 8. Phosphate buffer was used for these experiments. In subsequent experiments the pH of the reaction mixture was fixed to pH 6.0 throughout.

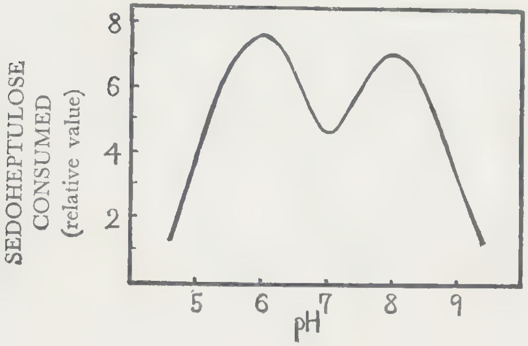

Fig. 2. Effect of pH on the sedoheptulose utilization. Phosphate buffer (*M*/20).

Necessity of Aerobiosis and Inorganic Phosphate—Fig. 3 shows the effect of anaerobic condition on the process. It is clearly indicated in the figure that the presence of molecular oxygen is required at least under the experimental conditions employed here. The implication of this oxidative character of the process will be discussed later.

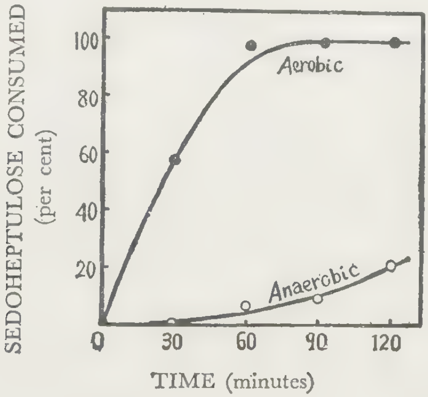

Fig. 3. Effect of anaerobic condition on the sedoheptulose utilization. Phosphate buffer, pH 6.0.

The indispensability of inorganic phosphate for the process was also suggested by the data presented in Fig. 4. If the usual phosphate buffer is replaced by veronal buffer of the same pH, a considerable delay in the utilization was observed. The use of veronal buffer, however, did not completely abolish the activity. This might be attributed to the endogeneous phosphate which had not been removed from the cells. In fact, it was later shown that the acetone-dried cells were almost completely inactive in veronal buffer.

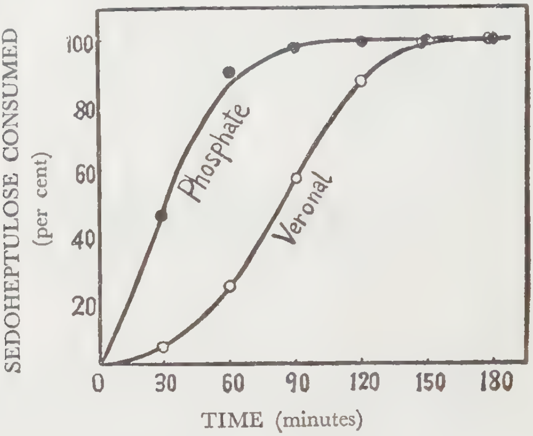

FIG. 4. Effect of phosphate and veronal buffers (both pH 6.0) on the sedoheptulose utilization.

Effect of Inhibitors—Since the findings desdribed above suggested the possibility that the sedoheptulose-utilizing mechanism may be closely associated with respiratory activity of the cells and some oxidative phosphorylation mechanisms may be involved in the process, the effect of inhibitors of either respiration or aerobic phosphorylation was studied. As shown in Table II, all the inhibitors tested exhibited remarkable inhibition upon the process. Especially, the marked inhibition produced by 2,4-dinitrophenol, a well-known inhibitor of oxidation-coupled phosphorylation, as well as that effected by arsenate, a disturber of a number of phosphate metabolism, made it decisive that the respiration-coupled phosphorylation is an integral part of the heptose untilization. Fluoride was strongly inhibitory with the acetone-dried cells, but failed to inhibit the washed cells. This might be due to inpermeability of the inhibitor into intact cell-walls.

Effect of Mg^{++}, Mn^{++}, and ATP—The above consideration that the oxidative phosphorylation is involved in the heptose utilization led to the assumption that ATP might be the key substance in the process.

If this is actually the case, the simplest possibility that accounts for all of the above results would be that the first step of the utilization be the phosphorylation of the heptose with ATP catalysed by a phosphokinase. In order to test the validity of this hypothesis, the effect of Mg^{++} and Mn^{++} as well as that of ATP were studied, since it is well known that all of the phosphokinases ever described are known to be highly activated by either Mg^{++} or Mn^{++} or by both (*19*). The acetone-dried cells were used for this purpose.

Table II

Effect of Inhibitors on the Sedoheptulose Utilization

Inhibitor	Final concentration	Inhibition per cent
	mole per lit.	
KCN	10^{-2}	100
NaN_3	10^{-2}	100*
2,4-Dinitrophenol	10^{-3}	100
Na_2HAsO_4	3×10^{-2}	100
NaF	10^{-2}	0
NaF**	4×10^{-2}	100
NaF**	4×10^{-3}	96

* Since NaN_3 interferes the sedoheptulose determination, this datum is only an approximation.

** The acetone-dried cells (20mg.) were used instead of the washed cells. $MgCl_2$ was also added to a final concentration of $10^{-3}\,M$. 14 mg. (dry weight) of the washed sedoheptulose-adapted cells were shaken with 560 μg. of the heptose in the presence of the inhibitors for 60 minutes. $M/20$ phosphate buffer, pH 6.0.

The experiments showed, as expected, that a remarkable increase in the heptose utilization was seen in phosphate buffer by the addition of Mg^{++}, whereas Mn^{++} exerted a considerable inhibition. These data are shown in Fig. 5 (A). Evidence in favour of the suggested " priming " of the heptose with ATP was further obtained by other experiments in which veronal buffer was used instead of phosphate buffer. The results of these experiments are presented in Fig. 5 (B). It will be seen that in veronal buffer little utilization takes place with the acetone-dried cells in contrast to the rather high activity of the intact cells in the same buffer (Fig. 4). This difference may be due to a relatively low content of endogeneous phosphate in dried cells. This fact also confirmed the above assumption that inorganic phosphate is necessary for the cells to actively utilize the heptose. It was further found, however, that if ATP is added to the system the cells can consume the heptose at a relatively rapid rate even in the absence of inorganic phosphate. This fact seems to provide a strong support for the hypothesis that ATP generated from inorganic phosphate by oxidative phosphorylation is actually the key substance of the heptose utilization. It became, therefore, more likely to accept the possibility that the utilization of the free heptose is initiated by a phosphokinase-catalysed phosphorylation of the sugar with ATP. Further indirect support for the functioning of such a phosphokinase was provided by the observation that the addition of Mg ($10^{-3}\,M$) to the ATP fortified dried cells further enhanced the utilization in veronal buffer.

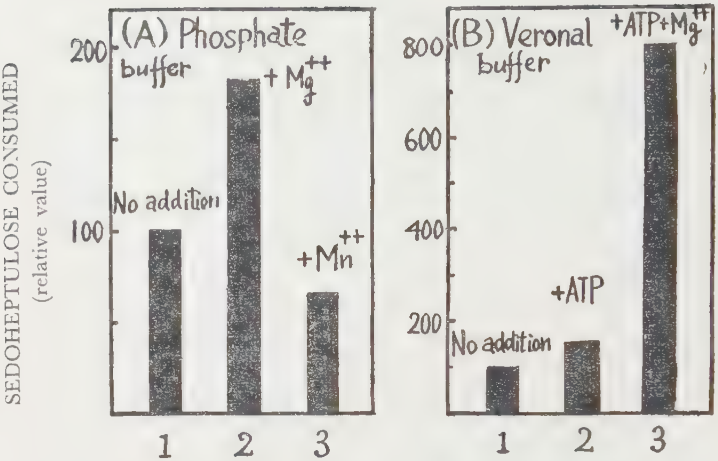

FIG. 5. Effect of Mg^{++}, Mn^{++} and ATP on the sedoheptulose utilization by dried cells. (A) Experiments in *M*/45 phosphate buffer (pH 6.0). 1. No addition; 2. 10^{-3} *M* $MgCl_2$ added; 3. 10^{-3} *M* $MnSO_4$ added. (B) Experiments in *M*/45 veronal buffer (pH 6.0). 1. No addition; 2. 10^{-3} *M* ATP added; 3. ATP, Mg^{++}, added (both 10^{-3} *M*).

DISCUSSION

Although a good deal of work is still required before we can be sure of the mechanisms of the heptose utilization by the adapted bacteria, it does not seem unreasonable to conclude from the results reported here that sedoheptulose must be first phosphorylated with ATP prior to further degradation or assimilation. This conclusion is also in good agreement with the recent knowledge of sedoheptulose metabolism (*1–3*, *5–9*) which shows that this sugar appears in active metabolism only in the form of phosphate ester. It may be reminded here that the hexose metabolism in various tissues and cells requires, in almost all cases, the preliminary activation of the sugars by introducing phosphate groups into the molecules. Furthermore, it has been revealed in recent years that some bacteria produce pentokinases (catalysing the phosphorylation of pentoses with ATP) in their cells when they are adapted to ferment several pentoses (*20*, *22*). The phosphorylation of glucosamine with ATP in brain tissue has been also reported as the first step of the hexosamine utilization (*22*). Taking all these findings into consideration, it would be understandable that our conclusion represents no more than the general tendencies of living systems to handle the substrates of monosaccharide nature.

In order to establish the above conclusion, however, it is of utmost importance to confirm the actual functioning of the postulated phosphokinases in the adapted cells and further to isolate this enzyme in a cell-free state. Unfortunately, our results are still rather rudimentary and inconclusive in these points. (Investigations along these lines are now in progress in our laboratory). The activation of the reaction by a low concentration of Mg^{++} and its inhibition by fluoride (with the dried cells), however, lend, though indirectly, some evidence for such an enzyme, since these characteristics are common features for all the phosphokinases (*19–21*). It may further be said that, if such a phosphokinase actually takes part in the process, this enzyme is different from the usual kinases acting on hexoses, since it can be formed only after adaptation to the heptose. No or little activity could be observed with the cells grown on glucose, fructose and sucrose.

As far as the present investigation is concerned, no information is as yet available concerning the chemical nature of the postulated phosphate derivative.

SUMMARY

1. Three strains of bacteria were isolated which utilize sedoheptuloe during growth.

2. The utilization was found to be due to the adaptive formation of an enzyme system in the cells.

3. Using the suspensions of the adapted cells (washed or acetone-dried), it was found that the presence of molecular oxygen and inorganic phosphate are required for the rapid utilization.

4. The process is inhibited by cyanide, azide, 2, 4-dinitrophenol, aresenate, and fluoride.

5. ATP can replace inorganic phosphate as a stimulant for the rapid utilization.

6. Mg^{++} enhances the process, whereas Mn^{++} is inhibitory.

7. It was concluded that the initial step of the utilization may be the phosphorylation of the heptose with ATP under the influence of a specific phosphokinase.

We are much obliged to Prof. T. Ishikawa and Dr. Y. Kurata of the Department of Pathology, Faculty of Medicine, Kanazawa University for their kindness in permitting us to use many of their instruments through all phases of this work.

REFERENCES

(*1*) Benson, A. A., Bassham, J. A., and Calvin, M., *J. Am. Chem. Soc.*, 73, 2970 (1951)

(*2*) Benson, A. A., Bassham, J. A., Calvin, M., Hall, A.G., Hirsch, J. E., Kawaguchi, S., Lynch, V. H., and Tolbert, N. E., *J. Biol. Chem.*, **196**, 703 (1952)

(*3*) Benson, A. A., Kawaguchi, S., Hayes, P., and Calvin M., *J. Am. Chem. Soc.*, **74**, 4477 (1952)

(*4*) La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, **30**, 61 (1917)

(*5*) Horecker, B. L., and Smyrniotis, P. Z., *J. Am. Chem. Soc.*, **74**, 2124 (1952)

(*6*) Horecker, B. L., and Smyrniotis, P. Z., *J. Am. Chem. Soc.*, **75**, 2021 (1953)

(*7*) Hough, L., and Jones, J. K. N., *J. Chem. Soc.*, **67**, 342 (1953)

(*8*) Robison, R., Macfarlane, M. S., and Tazelaar, A., *Nature*, **142**, 114 (1938)

(*9*) Ehrensvärd, G., 2^{e} Cong. intern. biochim., *Symp. sur metabolismes microb.*, p. 72 (Paris, July, 1952)

(*10*) Gilvarg, C., and Bloch, K., *J. Biol. Chem.*, **199**, 689 (1952)

(*11*) Sato, R., *Kagaku* (*Science*) (in Japanese), **23**, 474 (1953)

(*12*) Umbreit, W. W., in Umbreit, W. W., Burris, R. H., and Stanffer, J. F., "Manometric techniques and tissue metabolism," p. 218, New York 1951

(*13*) Nordal, A., *Arch. Pharm.*, **278**, 289 (1940)

(*14*) Partridge, S. M., *Nature*, **164**, 443 (1949)

(*15*) Klevstrand, R., and Nordal, A., *Acta Chem. Scand.*, **4**, 1320 (1950)

(*16*) Bevenue, A., and Williams, K. T., *Arch. Biochem. Biophys.*, **34**, 227 (1951)

(*17*) Szent-Györgyi, A., "Chemistry of muscular contraction," p. 143 New York 1951

(*18*) Nordal, A., and Klevstrand, R., *Anal. Chim. Acta*, **4**, 411 (1950)

(*19*) Colowick, S. P., in Summer, J. B., and Myrbäck, K., "The Enzymes" Vol. 2. Part 1. p. 128 (New York 1951)

(*20*) Hochster, R. M., and Walson, R. W., *Nature*, **170**, 357 (1952)

(*21*) Lampen, J. O., *Bacteriol. Rev.*, **16**, 211 (1952)

(*22*) Harpur, R. P., and Quastel, J. H., *Nature*, **164**, 693 (1949)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

PEPTIDES OF AMINOBENZOIC ACID AND THEIR HYDROLYSIS

IV. CONSECUTIVE HYDROLYSIS OF DIGLYCYL DIAMINOBENZOIC ACID

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(Received for publication, December 12, 1953)

Since Balls and Köhler (*1*) reported susceptibility of various aniline peptides to enzymatic hydrolysis, some peptides of aminobenzoic acid were used by several authors (*2*, *3*, *4*, *5*, *6*) to investigate whether the results could be explained by the rule discussed in those days concerning the relationship between specificity of peptidases and presence of free α-amino and/or α-carboxyl group adjacent to the sensitive peptide bond. However, later investigations on the specificity problem focused in general rather on the kind than number of constituting amino acids and since then those aniline peptides have been scarcely dealt with by enzyme researchers. The present studies on peptides of aminobenzoic acid had been taken up by us for practical purposes, as a minute amount of amino benzoic acid liberated could be estimated exactly by photometry. It was found thereby that glycyl and leucyl m-aminobenzoic acid were suitable substrates for investigating the difference of general and leucyl peptidases (*7*, *8*), and furthermore by employing DL-alanyl and DL-leucyl *m*-aminobenzoic acid the presence of a powerful D-peptidase in the autolysate of *Bac. subtilis* 016 was observed (*9*). The experiment presented below concerns a consecutive hydrolysis of diglycyl diaminobenzoic acid by animal enzyme. Either monoglycyl or free 3, 5-diaminobenzoic acid which would be produced successively can be estimated separately, hence kinetics of the consecutive reaction is ready for investigation.

EXPERIMENTAL

Diglycyl 3, 5-*Diaminobenzoic Acid* (*DG-DAB*)—Bis-(3, 5-chloracetyl)-diaminobenzoic acid recrystallized from 70 per cent alcohol was aminated by being left in concentrated ammonia and the residue dried *in vacuo* was extracted three times with hot water.

Monohydrochloride of DG-DAB in the extract was purified through repeated precipitation from its aqueous solution by the addition of 10 volumes of alcohol. The faintly yellow crystals obtained contained five moles of water of crystallization which was deprivable at 110° *in vacuo*. Kjeldahl-N of dehydrated hydrochloride, found 18.2 per cent; calculated 18.5 per cent: Cl found 13.5 per cent; calculated 13.3 per cent.

Monoglycyl 3, 5-Diaminobenzoic Acid (G-DAB)—3-Chloracetylamino-5-nitrobenzoic acid (m.p. 224°) recrystallized from 70 per cent alcohol was aminated and the product was purified by recrystallization from hot water. This nitrocompound (decomposition point 269°) was reduced to the coresponding amino derivative by warming with ammonium sulfide, the reaction mixture concentrated nearly to dryness *in vacuo*, and extracted by dilute ammonia. When the extract now free from sulfur was again concentrated *in vacuo*, crystals of G-DAB precipitated as thin plates, which were purified further by treatment with activated charcoal and recrystallization from hot water. This peptide contained 2 moles of water of crystallization which were deprivable at 110° *in vacuo*. Analysis of the dehydrated substance: N found 21.0 per cent, calculated 20.7 per cent.

Estimation of Free Diaminobenzoic Acid (DAB)—A color reaction for free DAB which should be negative against monoglycyl-DAB was found in a coupling reaction with diazo reagent at a weak acidity. This reagent was prepared as follows: To 10 ml. of cooled 0.01 *M* sulfanilic acid solution were added one drop of 30 per cent sodium nitrite, and 10 minutes later, 10 ml. of 1 *M* urea solution. The mixture was warmed at 30～40° for 20 minutes. For the estimation of free DAB, 2 ml. of the test solution was deproteinized by introducing 3 ml. of 5 per cent trichloracetic acid and to one ml. of the filtrate was added one ml. of *N* sodium acetate, whereby pH became about 5. One ml. of the diazo reagent was then added, one minute later the mixture was diluted to the 10 ml.-mark, and within 20～40 minutes thereafter the orange colored solution was photometrised by Pulfrich's apparatus using filter S_{47}. When the concentration of DAB in the original test solution was 0.0005 *M*, extinction coefficient E was 0.70. E was proportional to the concentration.

Estimation of Monoglycyl Diaminobenzoic Acid (G-DAB)—Principle of the method was similar, with a slight modification, to that of free *m*-aminobenzoic acid reported already (*5*). To 1 ml. of the deproteinized filtrate obtained as mentioned above were introduced, while cooling in ice water, 5 drops of *N* hydrochloric acid, one drop of 30 per cent sodium nitrite, and then 2 minutes later 2 ml. of the H-acid reagent which will be described below. The mixture was diluted with cold water to 10 ml. and after 20 minutes the red color of the solution was measured by photometry with filter S_{53}. Extinction coefficient was proportional to the concentration. When the original test solution contained G-DAB at 0.0005 *M* concentration, E was 0.49 after substracting a blank value. According to this procedure *m*-aminobenzoic acid of the same concentration gave an equal E, hence this compound was employed thereafter for calibration of E of G-DAB solution. The H-acid reagent used in this case was prepared on each measurement by mixing 2 ml. of 0.5 per cent H-acid (8-amino-1-naphthol-3,6-disulfonic acid) dissolved in 0.1 per cent sodium bisulfite, 5 ml. of 10 per cent ammonia, 4 g. of sodium sulfite, and 15 ml. of water. This reagent contained 3 g. more sodium sulfite than that used previously for estimation of *m*-aminobenzoic acid because a preliminary experiment indicated that thereby the dye formation out of G-DAB would decrease to 80 per cent but that of DAB could be inhibited so extensively that 0.0005 *M* DAB at original concentration gave such a low extinction coefficient as 0.04 in photometry after 20 minutes. This slight coloring of free DAB would, however, necessitate a correction of the G-DAB values observed, which was made as follows. Estimation by the diazo reagent of free DAB in the deproteinized solution was carried out simultaneously and the extinction observed was converted by calculation into that which the modified H-acid method would find in order to subtract it from the extinction value of the same solution measured by using the H-acid reagent as described above.

Enzyme Solutions—Kidney, pancreas and intestinal mucous membrane of pig were homogenized respectively with two volumes of water, and autolysed for 3 days at 37°. Each autolysate was then dialysed against running water for 2 days and stored in an ice chest. During autolysis, dialysis, and hydrolytic experiment some toluen was overlayed.

Composition of the test solution was 1 ml. of 0.01 *M* substrate solution, 3 ml. of 0.1 *M* buffer, 1 ml. of enzyme solution, and 5 ml. of water. The experiments were carried throughout at 37°.

RESULTS

Hydrolysis of DG-DAB at Various pH—When hydrolysis by pancreas autolysate was tested after incubation for 30 minutes, a remarkable liberation of G-DAB was observed, while free diamino acid (DAB) was scarcely detectable through the range of pH from 4 to 9. The pH curve of the amounts of G-DAB estimated was bell form (Fig. 1.) composed of symmetrical branches sloping from the maximum of 65 per cent at pH 7.2~7.6 towards both sides.

After incubation for 3 hours, however, the shape of pH curve changed extraordinarily as is illustrated in Fig. 1. A saddle-shaped G-DAB curve was here visible. Since the amount decreased of DG-DAB should be the sum of G-DAB and DAB estimated at the same time, hydrolysis of DG-DAB had attained 100 per cent at pH 7 and 8.

Comparison of this result by pancreas with that by intestine autolysate is illustrated in Fig. 2. which idicates remarkably that the intestine autolysate had produced already at the stage of 80 per cent hydrolysis of DG-DAB a fair amount of free DAB. Behaviour of kidney autolysate was similar to that of the intestine.

Hydrolysis of DG-DAB at pH 7.4—The amount of the enzyme solution to be added was so adjusted as either pancreas or kidney (used

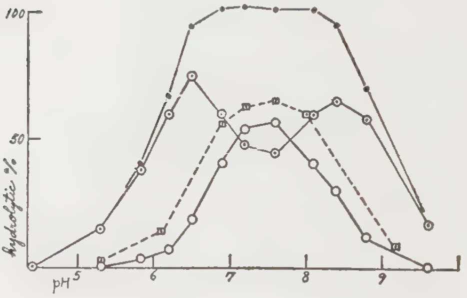

Fig. 1. pH Curves of DG-DAB hydrolysis by pancreas enzyme at 37° and various acidities. Buffer solution used is phosphate; pH above 8 and below 6 are adjusted by dilute ammonia and acetic acid respectively. Curve —▣-- is the result after 30 minutes incubation. DAB is not detectable. Results after 3 hours incubation are illustrated: —⊙— G-DAB; —○— DAB; —●— the sum of G-DAB and DAB, namely decreased amount of DG-DAB.

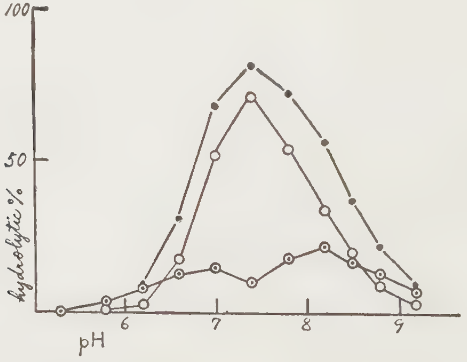

Fig. 2. pH Curve of DG-DAB hydrolysis by intestine enzyme measured after 3 hours' incubation at 37°. —⊙— G-DAB; —○— DAB; —●— the sum of G-DAB and DAB.

here instead of intestine) autolysate would hydrolyse DG-DAB nearly to 100 per cent in two hours. As seen in Figs. 3 and 4 the rise and fall of measurable amount of G-DAB distinctly differd from each other.

Hydrolysis of G-DAS by Kidney Autolysate—The rate of hydrolysis of G-DAB itself at pH 7.4 was compared with that of DG-DAB at the

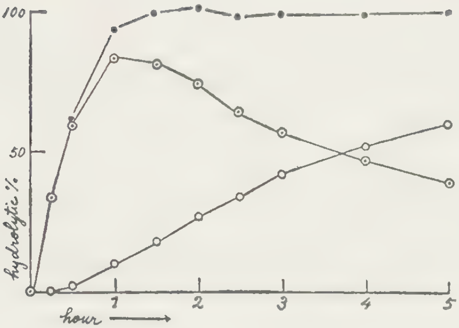

FIG. 3. Hydrolysis of DG-DAB by pancreas enzyme at 37° and pH 7.4 (phosphate buffer). —⊙— G-DAB; —○— DAB; —●— the sum of G-DAB and DAB. Maximum of G-DAB curve was attained when *x*/*A* was 83%. See text.

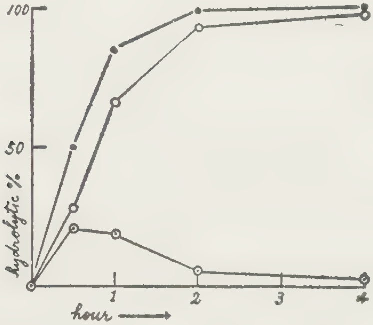

FIG. 4. Hydrolysis of DG-DAB by kidney enzyme at 37° and pH 7.4 (phosphate buffer). —⊙— G-DAB; —○— DAB; —●— the sum of G-DAB and DAB. Maximum of intermediate G-DAB production is attained when *x*/*A* was 20%. See text.

same final 0.001 *M* concentration. The enzyme solution employed was previously diluted five times; otherwise hydrolysis of G-DAB tested here as a primary substrate would go on too rapidly to be measured at given intervals of time.

DISCUSSION

Enzymatic hydrolysis of diglycyl diaminobenzoic acid (DG-DAB)

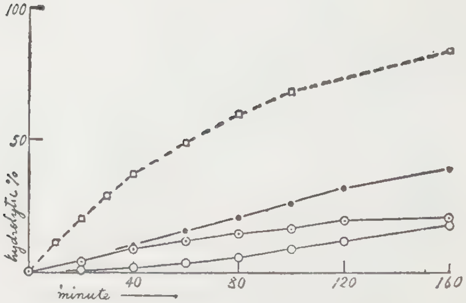

FIG. 5. Rate of hydrolysis by kidney enzyme of G-DAB and DG-DAB at equal initial concentrations. Hydrolysis of G-DAB: --□-- DAB. Hydrolysis of DG-DAB: —⊙— G-DAB; —○— DAB; —●— the sum of G-DAB and DAB. This figure shows also G-DAB curve attained maximum when x/A was nearly 20%.

proceeds in two steps. They are formation of monoglycyl diaminobenzoic acid (G-DAB) and its splitting to free diaminobenzoic acid (DAB) and glycine (G).

I. DG-DAB $\longrightarrow$ G-DAB + G
II. G-DAB $\longrightarrow$ DAB + G

In the case of pancreas the first reaction is faster than the second while in the case of kidney and intestine the relationship is reversed. Whether the two reaction were catalysed by two different enzymes is not definitely clear. However, it has been observed that when the kidney autolysate was acidified to pH 4 and the centrifuged supernatant was then warmed at 55° for 30 minutes, its G-DAB splitting component was inactivated more intensively than the other so that the warmed kidney autolysate behaved like that of the pancreas enzyme.

The hydrolysis of DG-DAB is an example of consecutive enzyme reaction and here quantitative estimation of G-DAB was possible; therefore, the maximum amount of this intermediate substance, which would accumulates during the reaction, was culculated under following considerations.

When each step of the reaction is of the first order, x amount of G-DAB to be measured at t time is given by equation 1, where k_1 and k_2 are the velocity constants of the reaction I and II, respectively, and A the initial concentration of DG-DAB, hence x/A is hydrolytic percentage.

$$\frac{x}{A}=\frac{k_1}{k_1-k_2}\left(e^{-k_2t}-e^{-k_1t}\right)\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\quad(1).$$

Through integration, one knows x/A would be the maximum, when

$$t=\frac{1}{k_1-k_2}\,ln\frac{k_1}{k_2}\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\quad(2).$$

At this t time,

$$\frac{x}{A}=\frac{k_1}{k_1-k_2}\left(e^{-\frac{k_2}{k_1-k_2}\,ln\frac{k_1}{k_2}}-e^{-\frac{k_1}{k_1-k_2}\,ln\frac{k_1}{k_2}}\right)\cdots\cdots\cdots\cdots\cdots\cdots\quad(3).$$

For obtaining the maximum value of x/A, equation 3 requires not k_1 and k_2 to be known separately but merely their ratio which can be calculated from Fig. 5 according to the law that the time elapsed till a definite degree of hydrolysis is in inverse proportion to the activity of an enzyme. In the experiment referred to that figure, where the concentration of both substrates was the same and an equal amount of enzyme solution was employed, the time corresponding to 30 per cent hydrolysis was 110 and 30 minutes. Therefore $k_1:k_2=3:11$. Through inserting these values in equation 3, the maximum x/A to be attained is 17 per cent which agrees nearly, though not exactly, with the experimintal 20 per cent (*cf.*, Figs. 4 and 5).

The above calculation has been made, however, on the assumption that each step of the reaction, would proceed by obeying monomolecular reaction. In fact it was realized during experiments with G-DAB of different initial concentrations, but in the case of DG-DAB the k_1 values calculated according to the formula of monomolecular reaction always increased slightly with time. Data are omitted to spare the space. It should be added here that although both K_m for DG-DAB and G-DAB had been determined respectively by virtue of the k_2 values measured directly and by the k_1 values obtained each through graphic extrapolation, yet they could not be utilized since the equations including those K_m's were not to be solved at all for attainment of the maximum. Furthermore, in the present case the initial velocities had by themselves not to do with the calculation of the maximum.

TABLE I

pKm of Enzyme Substrate Complex

	DG-DAB	G-DAB
Pancreas	3.8	2.3
Kidney	3.5	2.5

At a consecutive reaction it should be considered that the course of each reaction-step would be controlled in a complicated manner by the amounts of the primary substrate, the intermediate and final reaction products. It was found that glycine inhibited hydrolysis of neither DG-DAB nor G-DAB; the inhibition of DAB or G-DAB upon hydrolysis of DG-DAB was difficult to measure exactly.

On the other hand, since the second step of the reaction, when tested in an isolated system, was of the first order, pKm of the enzyme G-DAB complex would be equal to that of the same enzyme complex referring to DAB. Then it may be supposed that DG-DAB also has an affinity for the enzyme of the second step. Accordingly at the successive hydrolysis of DG-DAB the relative rate k_2 of the second reaction would be, owing to a competitive inhibition by DG-DAB present in the mixture, a little less than that which had been inserted into Eq. 3. Therefore, it is very probable that the calculated maximum of G-DAB production is elevated and nears the observed percentage.

Since it seems that no such analysis of consecutive enzyme reaction, as above mentioned, has been reported hitherto, the results of the present experiment have been discussed though the substrate was unnatural.

SUMMARY

Consecutive hydrolysis of diglycyl diaminobenzoic acid by pancreas, kidney and intestine enzymes was investigated. The reaction proceeded in two steps; the intermediate production of monoglycyl diaminobenzoic acid and its successive spliting to free diaminobenzoic acid. In the case of pancreas enzyme the rate of the first step-reaction was larger than the second. Therefore, the maximum amount of monoglycyl diaminobenzoic acid detected during the course of hydrolysis was remarkably different according to the enzyme used. The relative rates of two step-reactions were measured by employing their own substrates and that maximum amount to be detected was calculated, which agreed fairly well with the values observed experimentally.

REFERENCES

(*1*) Balls, A.K., and Köhler, F., *Ber. dtsch. chem. Ges.*, **64**, 34 (1931)
(*2*) Waldschmidt-Leitz, E., and Balls, A. K., *Ber. dtsch. chem. Ges.*, **64**, 45 (1931)
(*3*) Utzino, S., Z. *physiol. Chem.*, **198**, 135 (1931)
(*4*) Bergmann, M., Zervas, L., Fruton, J. S., Schneider, L., and Schleich, H., *J. Biol. Chem.*, **109**, 325 (1935)

(*5*) Grassmann, W., Klenk, L., and Peters-Mayer, T., *Biochem. Z.*, **280**, 307 (1935)

(*6*) Utsunomiya, T., *J. Biochem.*, **35**, 103 (1941)

(*7*) Furiya, S., *Symp. Enz. Chem.* (in Japanese), **4**, 64 (1950)

(*8*) Furiya, S., *J. Chiba Med. Soc.* (in Japanese), **29**, 124 (1953)

(*9*) Furiya, S., *J. Chiba Med. Soc.* (in Japanese), **29**, 129 (1953)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

NUTRITIVE VALUE OR TOXICITY OF HIGHLY UNSATURATED FATTY ACIDS. I

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(Received for publication, December 12, 1953)

According to the result from the experiments on the nutritive value of fish oils conducted by Ozaki (*1*) and Yoshida (*2*), highly unsaturated fatty acids in fish oils give toxic effects on the growth of rats. So far, this has been generally conceded, and the existence of highly unsaturated fatty acids in fish oils has been thought to account for the inferiority of fish oils to other edible oils from nutritive point of view. If this is actually the case, it might be surmised that eating of sardines, for example, in a large amount at a time or successively at short intervals would bring about harmful effects on our health.

It is a well known fact that highly unsaturated acids are rapidly oxidized when exposed to air. For this reason, a special attention must be paid when we carry out a feeding experiment by administrating highly unsaturated acids to test animals, and even if the experiment is started with genuine highly unsaturated acids, there will be a danger of supplying more or less autoxidized acids to the animals.

As has been reported in the previous paper (*3*), we examined the nutritive values of ethyl ester of highly unsaturated acids with iodine value of 365 and their partially reduced products. During the assay period the rats receiving ethyl ester of these acids showed a symptom of losing hairs around their mouths and legs. The ethyl ester used for the previous test was preserved in a brown bottle, and every time the bottle was opened, CO_2 gas was introduced, so that the acid might be kept from oxidation. In the later course of the experiment, however, we found that it was impossible to prevent the acid from deterioration by such a treatment as described above. Therefore, there was a possibility that we were measuring the nutritive value of the acid which was not so genuine as we thought to be, but which had become slightly oxidized. On the other hand, it was revealed by a successive experiment carried out by us that the partially reduced products of highly unsaturated acids, though they had fairly high iodine values, were almost as nutritious as oleic acid.

It has been known that natural unsaturated acids are to be easily oxidized when the number of the double bond increases, and the oxidation starts from the most unstable carbon existing in the molecules of unsaturated acids.. On the other hand, oleic acid may be regarded as one of the unsaturated acids of stable structure. These facts may suggest that the partially reduced products of highly unsaturated acids have become as nearly stable as oleic acid, dispite their fairly high iodine volues, when unstable factors have been removed from highly unsaturated acids by the reduction. And again because of the improved stability they became less oxidizable, and therefore, more nutritious than before.

The assumption has been further advanced that natural unsaturated acids, except one which has the conjugated double bond, would not always become less nutritious, as it has been generally believed, by the increase of double bond. In other words, unsaturated acids with the same number of carbon, even if they vary in degree of unsaturation, are almost equal in their nutritive values; and the long accepted differences among the nutritive values of unsaturated acids should certainly be attributable mainly to the degrees of the oxidation which is caused by the unstability of unsaturated acids. According to our opinion therefore, highly unsaturated acids such as contained in fresh sardines, without being oxidized, are not only harmless but also very nutritious for animals. Highly unsaturated acids which showed retarding effects in previous studies must have been those which became autoxidized to some extent.

In confirmation of this hypothesis, we extracted a sample of highly unsaturated acids as genuinely as possible from sardine oil and administered it to the rats, while oleic acid and palmitic acid were used as the control. Just as we expected, the rats did not show any distaste in taking the diet prepared with the unoxidized highly unsaturated acid, and they showed, from the nutritive point of view, almost as good results as those fed with oleic acid. They had a better result than those fed with palmitic acid. Whereas, the other groups of the animals which had received the diet containing highly unsaturated acids obtained from the same source, but oxidized by atmospheric oxygen, all perished after losing their weight and hairs. A slight deficiency in the nutritive value of the test acid in comparison to that of oleic acid might be explained by a supposition that the test acid was likely autoxidized a little in spite of our effort to keep it unchanged.

During the experiment the amount of yeast fed to the rats was made minimum so as to avoid the effect of vitamin B group upon them. The idea was to re-examine the view that the toxic effect of highly unsaturated acids on animals can be antidoted by giving a large quantity of riboflavin. However, the administration of that few amount of vitamin B did not seem to have ill effect on the animals fed with highly unsaturated acids which were not much oxidized. The fact led us to the belief that it might not be the acids themselves that riboflavin counteracts against, but the oxidized products which turned out of highly unsaturated acid. However, further research should be made in this respect.

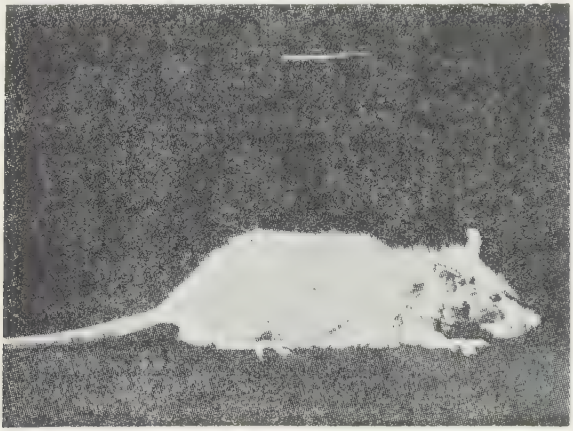

FIG. 1. A test rat fed with autoxidized ester of highly unsaturated fatty acid. Note depilated condition around the face which occurred in practically all of the test animals fed with the ester.

EXPERIMENTAL

A. Preparation of Fatty Acids—Bromination method usually applied to separate highly unsaturated acids was not feasible for the present purpose, because it was thought that bromination of polyethenoid fatty acid would result in forming two or more isomeric bromo derivatives. The authors, therefore, carried out the separation by low temperature crystallization method, of which procedures are as follows:

Extract oil from fresh sardine by boiling to saponify it with alcoholic sodium hydroxide solution, and acidify by adding HCl. The fatty acids thus obtained are washed with hot water, then dehydrated. After adding acetone ten times as much as the quantity of the mixed fatty acids, keep them standing in a cold storage overnight to filtrate solid acids crystallized from the solution and remove unsaponified matters with ether. Apply sodium salt-acetone method twice to the above mixed acids to obtain the crude product of highly unsaturated acids, which will be changed into ethyl ester in the presence of HCl-catalyst, and subjected to the vacuum distillation (2 mm, 190–200°). The sample, when deodorized by blowing H_2 gas into it at 80–100° for 30 minutes, will bear a slightly yellowish tinh. It is recommended to store the sample in a thermos with crushed dry ice so as to prevent it from autoxidation.

The sample of oxidized acids was prepared by leaving a 4 mm-layer of genuine highly unsaturated acids in an open basin at room temperature. One of the controls, ethyl ester of oleic acid was prepared from mixed fatty acids of camellia-oil by lead salt-alcohol method, and the other, ethyl ester of palmitic acid, by vacuum distillation of the commercial product.

B. Feeding Procedures—Weanling male and female rats obtained from our stock colony were depleted by feeding them with 9.5 g. of the fat-deficient diet per day per rat. When the weight remained constant over two weeks, they were separated into different groups, each consisting of the same sex of rats in an approximately similar number and weight. The supplements of unsaturated acids were daily administered 0.5 g. per rat over a 30-day period to observe growth and other physiological conditions of the animals.

Daily doses of vitamin B contained in beer yeast of the basal diet were 14.2 γ of thiamin and 11.4 γ of riboflavin per rat. Ingredients of the basal diet are given in Table I, and properties of the supplemented unsaturated acids in Table II.

TABLE I

Ingredients of Diets

	Basal diet	Test diet
Polished rice powder	79%	79%
Casein (Ether extracted)	10	10
Dried beer yeast	3	3
McCollum salts mixture	3	3
Ethyl ester of fatty acid	0	5
	95	100
Liver oil of tuna (1% ethanol soln.)	1 drop/day	

TABLE II

Analysis of the Samples Used in the Experiments

	Iodine value	Sap. value	Unsap. matter(%)
Ethyl ester of highly unsaturated acid	335.92	173.36	Trace
Oxidized ester of the above acid	161.08	——	Trace

C. Feeding Results—

(1) The results of the feeding experiment on genuine highly unsaturated acids and their oxidized product are summarized in Table III.

TABLE III

Increase of body Weight when the Samples Added

(Period: From September 9 to October 10, 1952)

Type of fat fed	Brood series	Sex	Weight gained in 32 days	Average gain in weight
Fat-free basal	Z D	♂ ♂	8g. 6	7g.
Ethyl ester of highly unsaturated acid	Z A B D E	♂ ♂ ♂ ♂ ♂	11 11 12 20 13	13.4
Oxidized ester of the above acid	Z A B D E	♂ ♂ ♂ ♂ ♂	–30 (died on 9th day) –53 (died on 11th day) –46 (died on 11th day) –47 (died on 23rd day) –42 (died on 11th day)	

Of the rats those which received unoxidized highly unsaturated acids exhibited no dislike in taking the supplemented diet. The others fed with the oxidized acids in the assay period manifested remarkable decrease in weight and the depilation. Most of them could not live longer than two weeks after the administration.

(2) In confirmation of these results, the assay was repeated over a 3-week period, using oleic acid and palmitic acid as the control. Properties of the supplements used and the data on the weight gained in the second assay are given in Tables IV and V, respectively.

TABLE IV

Analysis of the Samples Used in the Experiments

	N_D^{20}	Iodine value	Sap. value	Unsap. matter (%)
Ethyl ester of oleic acid (control)	1.4440	82.70	184.25	0
Ethyl ester of palmitic acid (control)	1.4348	0	196.20	0
Ethyl ester of highly unsaturated acid (Original free fatty acid)	1.4798	328.34 (353.87)	173.06	Trace
Slightly oxidized ester of the above acid	1.4800	312.40	——	Trace
Oxidized ester of the above acid	1.4854	285.74	171.20	Trace

TABLE V

Feeding Records

(Period: From December 9 to 31, 1952)

Type of fat fed	Brood series	Sex	Weight gained in 23 days			Average gain in weight
			At the end of 1st week	At the end of 2nd week	Last day	
Fat-free basal	F	♂	−2*g.*	−2*g.*	4*g.*	5.25 *g.*
	G	♂	−2	1	9	
	H	♂	−3	−2	5	
	H	♂	−7	−5	3	
Ethyl ester of oleic acid (control)	F	♂	3	14	23	21.5
	G	♀	3	9	22	
	H	♀	2	7	19	
	H	♂	0	9	22	
Ethyl ester of palmitic acid (control)	F	♂	2	5	15	13
	G	♀	0	4	12	
	G	♂	−1	4	13	
	H	♀	0	4	12	
Ethyl ester of highly unsaturated acid	F	♀	2	12	22	19.25
	G	♀	0	10	20	
	G	♂	1	5	18	
	H	♀	−4	5	17	
Slightly oxidized ester of the above acid	F	♀	0	7	18	15.25
	G	♀	−3	0	12	
	G	♂	1	9	16	
	H	♀	−3	5	15	
Oxidized ester of the above acid	F	♀	−6 (died on 4th day)			
	G	♀	−7 (died on 7th day)			
	G	♂	−6 (died on 6th day)			
	H	♀	−8 (died on 7th day)			

As it is obvious from Table V, the nutritive value of highly unsaturated acids was better than that of palmitic acid used as control, and not very much lower than that of oleic. In other words, the administration of unoxidized unsaturated acids was found to have no depressing effect on the growth of rats. Howeve, the autoxidized acid (iodine value 285) obtained from the test acid had such a seriously toxic effect on the rats that they all lost their lives in the first week of the assay period.

In addition, the coefficient of digestibility of each supplement has been determined by chemical analysis of the fecal matter. For this purpose, the feces of each group were collected daily for two weeks of the test period and stored in ethyl alcohol. In Table 6 are presented the data obtained from the fecal fat analized by an ordinary method.

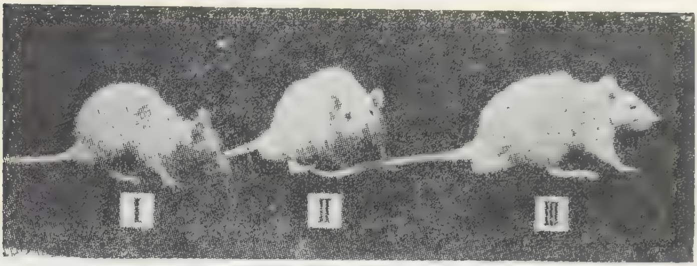

FIG. 2. Test rats, each fed with different type of ester: Oxidized ester of highly unsaturated acids for I, Ethyl ester of genuine highly unsaturated acids for II, Ethyl ester of oleic acid (control) for III.

TABLE VI

Analytical Results of Feces Excreted in Two Weeks

Type of fat fed	No. of rats	Total fat consumed	Total fat recovered from feces	Crude fat in feces	Unabsorbed fat from feces	Coefficient of digestibility	Iodine value of fecal fat
Fat-free basal	4	*g.	1.0412g.	2.31 %	*g.	*%	59.81
Et-ester of oleic acid (control)	4	28	1.9950	4.52	0.9538	96.59	74.92
Et-ester of highly unsaturated acid	4	28	2.6335	6.79	1.5923	94.31	160.75
Slightly oxidized ester of the above acid	4	28	4.1735	9.01	3.1323	88.81	74.92

* Not applicable.

a. The correction for metabolic fat was made by subtracting 1.04 g., the metabolic fat recovered in fat-free group, from each of the other values.

b. Coefficient of digestibility as used here was defined as that fraction of total ingested fat which was retained.

In respect to the coefficient of digestibility, genuine highly unsaturated acids were nearly the same as oleic acid. However, the iodine value of fecal fat of the former acid was higher than that of the latter.

Immediately after the experiment, the rats were put to death to examine thiamine and riboflavin contained in their livers.

It can be seen in Table VII that no special differences were obvious in the vitamin contents recovered from each group of the rats fed with different fatty acids.

Table VII

Vitamin B Contents in the Livers after Feeding Experiments

Type of fat fed	Vitamin B found in liver (γ/100 *g.*)	
	Thiamine	Riboflavin
Ethyl ester of oleic acid (control)	171	614
Ethyl ester of genuine highly unsaturated acid	160	634

DISCUSSION

Based on the above results, we recognized that highly unsaturated acids, when not oxidized, have a sufficiently high nutritive value for the growth of rats, and that it is not necessary to supplement the diet with a large quantity of yeast or riboflavin as it was believed to be. However, oxidized fatty acids are definitely inferior to genuine highly unsaturated acids in the nutritive value so that the administration of the former causes depilation to the test animals and even the death in the end. These evidences led us to a skepticism that most of the previous reports on the nutritive value of highly unsaturated acids might have been based on the acids which had been more or less oxidized.

It should be noted in this connection that the information obtained so far is not valid in explaining why oxidized highly unsaturated acids show an extremely retarding effect on the growth of animals. What we know at the present time is that hydroxy acids such as ricinoleic acids and dioxystearic acid are not nutritious. There are, however, only few reports on the oxidation of highly unsaturated acids which are supposed to be helpful in clarifying the results attained by our assay. The important among them are those which deal with rancid lard.

According to Rusoff and Hanson (*4*), the administration of rancid lard causes the biotin deficiency to rats, as the results that the biotin composition in the intestines of the rats is disturbed by the oxidized products. Kaunitz and his co-workers (*5*) pointed out that the diet containing rancid lard tends to cause the riboflavin deficiency to animals and the increase in requirements of various essential factors. Those facts seem to suggest something which may throw light on the results of our assay.

It has also been brought to our attention that the polymerized fish oil, depending on the treatments given, sometimes turns out highly nutritious, and indeed better than the original sample, and that there are considerable differences in the nutritive value between autoxidized products of highly unsaturated acids and the oxidized acid produced in the course of polymerization. Further studies will be made in respect to these differences.

SUMMARY

It has been widely accepted that the nutritive value of unsaturated fatty acids is depleted when the number of double bond in the acids increases. In order to prove whether this is true or not, we have carried out the following experiments, using the diet consisting of highly unsaturated fatty acids.

1. When the diet, containing 5 per cent of ethyl ester of highly unsaturated fatty acids kept from the oxidation as much as possible, was administered to the test rats together with a small amount of yeast (3 per cent), the normal growth of the rats was attained without showing retarding effects on their health. The nutritive value of the test ester was not much lower than that of oleic acid used for the comparison.

2. The rats, which were fed with the acids obtained from the same source and autoxidized, exhibited a retarding effect upon their growth with the decrease in weight and the depilation around their mouths and legs. They all died in the later course of the test.

On the basis of these assays, we have come to the conclusion that highly unsaturated fatty acids contained in fresh fish will not be harmful at all for the health of animals.

We hereby highly appreciate the helpful advices given by Dr. Jun'ichi Ozaki, and the constant encouragement by Dr. Hideo Higashi. We are also much indebted to the unselfish devotion of Misses Hisae Sakurai and Kimie Arai in the feeding of animals.

REFERENCES

(*1*) Ozaki, J., *J. Agr. Chem. Soc. Japan*, **8**, 1286 (1932)
(*2*) Yoshida, M., *J. Agr. Chem. Soc. Japan*, **13**, 120 (1937)
(*3*) Kaneda, T., and Ishii, S., *J. Jap. Soc. Food Nutrition* (in Japanese) **5**, 78 (1952)
(*4*) Rusoff, H., and Hanson, T., *Arch. sci. physiol.*, **2**, 313 (1948)
(*5*) Kaunitz, H., Johnson, R. E., and Slanetz, C. A., *J. Nutr.*, **46**, 151 (1952)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

MEASUREMENT OF GLUCURONIDE SYNTHESIS BY TISSUE PREPARATIONS

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(Received for publication, December 14, 1953)

The study of the biological mechanism of glucuronide synthesis requires a sensitive reaction, specific for glucuronide; for the measurement of the ability of surviving tissue slices to form glucuronides, three methods based on different principles have been devised. All of them, however, bear serious disadvantages and are inconvenient for routine works.

Thus in the method originated by Lipschitz and Bueding (*1*), the hard solubility of glucuronide in ether and the lack of specificity in the colour reaction (Tollens test) cause serious difficulties in their procedure, as Levvy and Storey have pointed out.

The next method, initiated by Levvy and Storey (*2*), may estimate the aggregate amount of both glucuronide and ethereal sulphate, as pointed out by Bernstein (*3*).

The third due to De Meio and Tkacz (*4*), is suitable for estimate ethereal sulphate, but it takes a long time to obtain the fraction of phenol bound as glucuronide and because of the variable recovery of phenol after treatment with $10N$ H_2SO_4, the method is certainly less advantageous for the analysis of the mechanism of glucuronide synthesis.

Here we may get a clue form the experiment of anthranilic acid glucuronide, which was undertaken by Shirai, one of the present authors, and Sasaoka (*5*, *6*) under the supervision of Prof. Kotake; the followings are a brief description of this experiment.

Whilst free anthranilic acid gave paperchromatogrophically a spot at Rf. 0.86 (solvent; N-butanol: acetic acid: water=4:1:1), the urine of rabbit, administered with anthranilic acid subcutaneously, gave a strong spot at Rf. 0.40 and two faint ones at Rf. 0.86 and 0.72. All these spots were detectable by Bratton and Marshall's diazo test (*7*). After the urine treated with 0.1 N NaOH at 55° for 60 minutes, the substance, which gave the spot at Rf. 0.40 disappeared, and the one at Rf. 0.86 augmented markedly, meanwhile a substance newly appeared, which gave a spot at Rf. 0.20, detectable by reducing test. This corresponds to free glucuronic acid.

From these observations, we may infer that the substance which gave the spot at Rf. 0.40 must be anthranilic acid glucuronide, and the carboxyl group of anthranilic acid and the acetal-OH of glucuronic acid must participate in conjugation, forming a glucuronide of ester-type.

Such inference would give a measure for the quantitative estimation of anthranilic acid glucronide, and a new method has been devised by which the study of glucuronide synthesis by tissue preparations could be followed.

EXPERIMENTAL

I. Colour Reaction of Anthranilic Acid

The diazotization of the acid and coupling with β-diethylaminoethyl-α-naphtylamine were carried out as described by Bratton and Marshall. To 10 ml. of the test solution was added 0.5 ml. of 4 *N* HCl, followed at suitable intervals by 1 ml. of each of the solutions: 0.1 g./dl. $NaNO_2$, 0.5 g./dl. ammonium sulphamate, and 0.1 g./dl. β-diethylaminoethyl-α-naphthylamine oxalate. After all the reagents were added, the test-tubes were immersed in a water bath kept at 37° for 3 hours. Then the extinction was measured using a filter S. 55.

II. Quantitative Separation of Free and Conjugated Anthranilic Acid as Glucuronide Formed by Rat Liver Slices

Into 5 ml. conical flasks fitted with rubber stoppers were introduced each 4 ml. phosphate Ringer's solution (modified by Krebs and Henseleit (*8*) and a 200 mg. slice. The solution here used contained 1×10^{-4} *M* anthranilic acid, but no sulphate, $MgSO_4$ being replaced by an equivalent concentration of $MgCl_2$.

After passing a brisk stream of pure O_2 for 1 minute, they were stoppered and shaked in the water bath kept at 37.5° for 90 minutes. The slices were then removed and the flasks were cooled in cold water for few minutes. 6 ml. of water and 2 ml. of 1 *M* trichloracetic acid were added to each flask. After 15 minutes the contents were filtered. 8 ml. of the filtrate was made faintly acid to congo red by adding 2 ml. of 0.5 *M* Na_2CO_3 and extraction by 15 ml. of ethyl ether was repeated three times. The aqueous layer was preserved for the determination of conjugated anthranilic acid as glucuronide. The total ethereal extracts were re-extracted twice by 4 ml. of 0.1 *N* NaOH. After the two alkaline extracts were mixed together and neutralized by 1 *N* HCl, the total was made up just to 10 ml. and the colour reaction was carried out as described above (*Fraction 1*).

The aqueous layer was made alkaline by adding 2 ml. of *N* NaOH and digested in the water bath at 55° for 60 minutes. After neutralized by N HCl, the contents were extracted by ethyl ether, the ethereal extracts were re-extracted by 0.1 N NaOH, and the colour was developed following the process (*Fraction 2*).

Fraction 1 contains free anthranilic acid and *Fraction 2* contains this acid derived from glucuronide.

III. Errors Involved

To test the extraction procedure 4 ml. of $1\times10^{-4}M$ anthranilic acid solution was treated as described above. The mean recovery in twelve observations was 98.4 per cent, with the largest deviation from the mean being 1.5 per cent. Neither the alkaline digestion affected the recovery least, nor the procedure of protein precipitation by trichloracetic acid caused any hydrolysis of anthranilic acid glucuronide. The latter was confirmed by the treatment of urine of rabbit administered subcutaneously with anthranilic acid. The separation of free anthranilic acid and conjugated anthranilic acid as glucuronide by these procedures seemed to be perfect judging from the observations made by paperchromatographic technique.

IV. Identification of the Compound Giving the Colour Reaction

We have assumed that the *Fraction 2* contains anthranilic acid derived solely from glucuronide. To confirm this point, we have tested the following compound: aniline, acetanilide, 5-hydroxyanthranilic acid (this compound was proved to be formed from anthranilic acid in animal tissue by Shirai (*9*)), and *N*-acetylanthranilic acid. None of these, however, appears in the *Fraction 2* to interfere with the assay of conjugated anthranilic acid as glucuronide.

The substance, which was excreted in the urine of the rabbits administered subcutaneously with anthranilic acid and gave the spot at Rf. 0.75 paperchromatographically (this was assumed as o-aminohippuric acid by Kikkawa (*10*)), is partially soluble in ether, so that this may interfere with the assay even though the extent of the disturbance thus caused may be negligible. Fortunately the substance is not formed from anthranilic acid in rat tissue.

DISCUSSION

The *Fraction 2* contains complete anthranilic acid solely derived from glucuronide, as the substance positive to Bratton and Marshall's diazo test. Due to the high sensitivity of this colour reaction, minute amounts of anthranilic acid glucuronide can be estimated with a recovery rate near 100 per cent.

Because of its specificity and sensitivity, the method described here seems highly convenient for the measurement of glucuronide synthesis by tissue preparations.

SUMMARY

1. Minute amounts of anthranilic acid can be determined colorimetrically after diazotizing and coupling with β-diethylaminoethyl-α-naphthylamine.

2. Free anthranilic acid and conjugated anthranilic acid as glucuronide can be determined separately by the present method.

3. The method has been applied to the measurement of glucuronide synthesis by rat-tissue slices.

It is our pleasurable duty to thank Prof. H. Mabuti for his continued interest and advices given during the course of this investigation.

REFERENCES

(*1*) Lipschitz, W. L., and Bueding, E., *J. Biol. Chem.*, **129**, 333 (1939)
(*2*) Levvy, G. A., and Storey, I. D. E., *Biochem. J.*, **44**, 295 (1949)
(*3*) Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **198**, 195 (1952)
(*4*) De Meio, R. H., and Tkacz, L., *J. Biol. Chem.*, **195**, 175 (1952)
(*5*) Shirai, Y., *et. al.*, Proc. 24th. Japan. Biochem. Soc., *J. Biochem.*, **39**, Proceedings, xxxxiv (1952)
(*6*) Sasaoka, T., *J. Wakayama Med. Soc.* (in Japanese), **2**, 103 (1951)
(*7*) Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, **128**, 537 (1939)
(*8*) Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 36 (1932)
(*9*) Shirai, Y., *J. Japan. Biochem. Soc.*, **22**, 223 (1950)
(*10*) Kikkawa, H., *Advances in Gene.*, **5**, 107 (1953)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

SYNTHESIS OF GLUCURONIDES BY TISSUE SLICES. I

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(Received for publication, December 14, 1953)

Although the formation of conjugated glucuronides in the animal body has been known for many years, little has been gained, however, on the chemical mechanism involved therein. The extensive investigations of conjugation process in isolated tissue preparations were carried out by Lipschitz and Bueding (*1*), Levvy and Storey (*2*, *3*), De Meio and Tkacz (*4*), and Bernstein and McGilvery (*5*). Yet the results produced by these authors, were rather variable, and the discrepancy may mainly be due to certain inefficaciousness of the methods employed.

In the present paper, the synthesis of glucuronide as it occurs in rat-tissue slices has been examined, using a specific, sensitive and rapid method developed by us and reported in the previous paper (*6*).

EXPERIMENTAL

Adult stock rats of either sex were used in all the experiments, and were well fed except where stated otherwise. The rats were killed by the cut of carotis under ethereal narcosis and the liver or other tissues were rapidly removed and cooled in crushed ice. Slices were cut into chilled phosphate Ringer's solution modified by Krebs and Henseleit (*7*), in which $MgSO_4$ was replaced by $MgCl_2$. The slices, wiped out of excess solution on filter paper, were weighed with a torsion-balance. The next procedures, namely the incubation of slices with anthranilic acid, protein precipitation, fractionation and colour development were carried out as described in the preceding paper.

RESULTS

Comparison of Glucuronide Formation by Different Tissues of the Rat—As is generally recognized, the liver was found to be a powerful organ in which the glucuronide is produced. This process took place also in kidney slices, but its speed was no more than that found in the liver. Surprisingly, the intestines or mesenterium showed a marked activity; especially, in the duodenum more glucuronide was produced than in the liver. A number of other tissues were examined, but all were found ineffective, although these were tested under optimal conditions (Table I).

TABLE I

Comparison of Glucuronide Formation from Anthranilic Acid by Different Tissues

(Concentration of anthranilic acid, $1 \times 10^{-4}M$)

Exp.	Organ	Anthranilic acid conjugated per cup				A/B
		By this organ (A)		By the liver of the same animal (B)		
		μg.	%*	μg.	%*	
1	Kidney	11.3	21	15.2	28	0.74
2	Spleen	0	0	7.7	15	0
3	Blood	0	0	11.6	22	0
4	Heart (muscle)	0	0	13.1	25	0
5	Skelet muscle	0	0	8.6	16	0
6	Lung	0	0	12.1	24	0
7	Testis	0	0	12.1	24	0
8	Cerebrum	0	0	7.8	15	0
9	Eyeball	0	0	13.1	25	0
10	Stomach	0	0	7.8	15	0
11	Duodenum	18.5	34	12.1	24	1.51
12	Ileum	7.2	13	7.8	15	0.91
13	Colon	6.7	12	7.8	15	0.85
14	Mesenterium	6.3	11	10.4	19	0.61

* Note: percentages are given with regard to the incubated anthranilic acid quantities.

Attempts to Stimulate Glucuronide Synthesis with Liver Slices—As shown summarily in Table II, glucose or fructose showed an inhibitory action. Glucuronate, with or without adenosinetriphosphate (ATP), inhibited the glucuronide synthesis. Lactate or pyruvate has a slight though perceptible activation. Both glycogen and glucose-1-phosphate activated the glucuronide synthesis in the highest rate.

DISCUSSION

With liver slices from guinea pigs, Lipschitz and Bueding (*1*) found that glucuronide formation was strongly augmented in the presence of lactate or pyruvate, quite opposite to Storey (*3*), who found that these compounds were ineffective.

In the present experiments, these compounds activate the glucuronide synthesis in the liver slices slightly, but to a recognizable degree. This indicates that these compounds are situated somewhere, probably far

TABLE II

Effects of Carbohydrates, 3-carbon Compounds or ATP on the Conjugation of Anthranilic Acid by the Liver Slices of Normal Rats

No.	Saline containing anthranilic acid and		Activation (+) or inhibition (−)
		M	%
1	Glucose	1×10^{-1}	−52.3
2	〃	2×10^{-2}	−35.1
3	〃	5×10^{-3}	−19.6
4	Fructose	2×10^{-2}	−26.3
5	Lactate	2×10^{-2}	+10.5
6	Pyruvate	2×10^{-2}	+ 2.8
7	Glycogen	1×10^{-1}	+10.4
8	〃	2×10^{-2}	+58.2
9	〃	5×10^{-3}	+11.3
10	Glucose-1-phosphate	1×10^{-1}	+15.5
11	〃	2×10^{-2}	+48.3
12	〃	5×10^{-3}	+ 0.8
13	Glucuronate	2×10^{-2}	−55.1
14	〃	5×10^{-3}	− 8.9
15	〃	5×10^{-4}	− 1.9
16	〃	1×10^{-4}	+ 0.9
17	(16)+ATP	1×10^{-4}	− 1.4
18	(16)+ATP	2×10^{-4}	−20.7
19	ATP	1×10^{-4}	± 0
20	〃	5×10^{-3}	−14.4

from glucuronide, on the metabolic pathway in the formation of glucuronide.

Bernstein and McGilvery (*5*) found that phosphorylated hexoses (glucose-1-phosphate and fructose-1,6-diphosphate) did not induce any synthesis. In our experiments glucose-1-phosphate is as active as glycogen, and both far exceed lactate and pyruvate in their activities. It may be possible to assume that the glucuronide synthesis proceeds *via* glucose-1-phosphate and a hypothetical glucuronate-1-phosphate.

Lastly the finding that a large amount of glucuronide is formed in the whole system of the intestines (see the last part of Table I), may be an interesting observation in the physiology of the intestines.

SUMMARY

1. A general survey on the sites of the glucuronide synthesis inside the body was made, and the liver, kidney, mesenterium and especially the intestines were found to be quite active.

2. The synthesis of glucuronide by rat-liver slices was augmented

strongly in the presence of glycogen and glucose-1-phosphate, and slightly by lactate and pyruvate.

It is a great pleasure to acknowledge our indebtedness to Prof. Mabuti for the interest shown and the advices given to us during the course of this investigation.

Addendum: Lately, a substance, which is assumed as α-glucuronic acid-1-phosphoric acid, was synthesized by us. The substance showed higher activity in glucuronide synthesis than did Cori's ester or glycogen. The details of the experiment will be reported soon after.

REFERENCES

(*1*) Lipschitz, W. L., and Bueding, E., *J. Biol. Chem.*, **129**, 333 (1939)

(*2*) Levvy, G. A., and Storey, I. D. E., *Biochem. J.*, **44**, 295 (1949)

(*3*) Storey, I. D. E., *Biochem. J.*, **47**, 212 (1950)

(*4*) De Meio, R. H., and Tkacz, L., *J. Biol. Chem.*, **195**, 175 (1952)

(*5*) Bernstein, S., and Mc Gilvery, R. W., *J. Biol. Chem.*, **198**, 195 (1952)

(*6*) Shirai, Y., and Ohkubo, T., *J. Biochem.*, **41**, 381 (1954)

(*7*) Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 36 (1932)

The Journal ot Biochemistry, Vol. 41, No. 3, 1954

ON THE STRUCTURE OF LYSOZYME

II. CHARACTERIZATION OF ASPARTYL, ASPARAGINYL, AND GLUTAMINYL RESIDUES IN LYSOZYME

By KO OHNO

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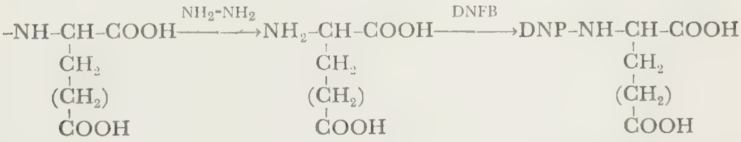
(Received for publication, December 14, 1953)

It has been difficult to examine, except certain special cases, how many aspartic and glutamic residues exist in a protein as one carboxyl-free or as amides, although the number of each acidic amino acid residues and of amide nitrogen can be analyzed quantitatively. Recently, Jollès and Fromageot (*1*) and Chibnall and Rees (*2*) have reported the method to characterize the mode of linkage of acidic amino acids in insulin, in which the protein is treated with reducing agents, *i.e.*, lithium aluminium hydride or lithium boron hydride, and, after hydrolysis, either the corresponding amino-hydroxy acids derived from aspartyl or glutamyl residues are examined (*1*), or residual amino acids not affected by the reagent are analyzed (*2*).

The author has reported the hydrazidolysis-DNP method, a method for quantitative determination of carboxyl-terminal amino acids in proteins, by which lysozyme has been proved to possess one leucine residue per mole at its carboxyl end (*3*). This method can be modified to characterize the mode of linkage of acidic amino acid residues in peptides. The possible changes of these residues by hydrazinolysis and dinitrophenylation are as follows:

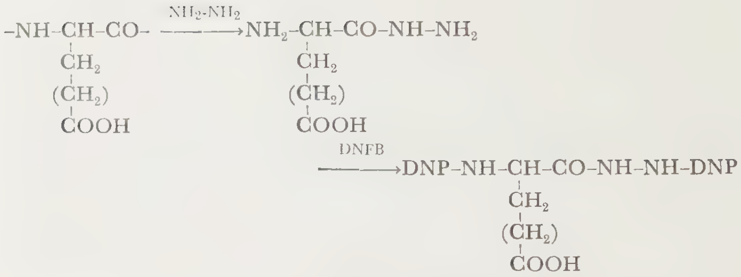
(a) Carboxyl-terminal as two carboxyl-free.



$$\begin{array}{l}\text{-NH-CH-COOH}\\ \qquad|\\ \qquad CH_2\\ \qquad|\\ \quad(CH_2)\\ \qquad|\\ \quad COOH\end{array} \xrightarrow{NH_2\text{-}NH_2} \begin{array}{l}NH_2\text{-CH-COOH}\\ \qquad|\\ \qquad CH_2\\ \qquad|\\ \quad(CH_2)\\ \qquad|\\ \quad COOH\end{array} \xrightarrow{\text{DNFB}} \begin{array}{l}\text{DNP-NH-CH-COOH}\\ \qquad\qquad|\\ \qquad\qquad CH_2\\ \qquad\qquad|\\ \qquad\quad(CH_2)\\ \qquad\qquad|\\ \qquad\quad COOH\end{array}$$

DNP-aspartic acid (Ia)
DNP-glutamic acid (IIa)

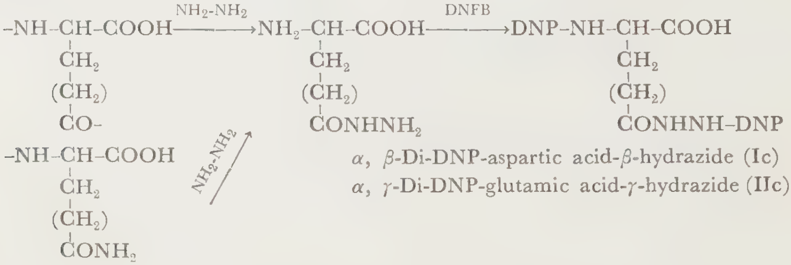
(b) α-Aspartyl or α-glutamyl residues in peptide,



$$\text{-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ COOH}}{CH}\text{-CO-}\xrightarrow{NH_2\text{-}NH_2}NH_2\text{-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ COOH}}{CH}\text{-CO-NH-NH}_2\xrightarrow{DNFB}\text{DNP-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ COOH}}{CH}\text{-CO-NH-NH-DNP}$$

α, α-Di-DNP-aspartic acid-α-hydrazide (Ib)
α, α-Di-DNP-glutamic acid-α-hydrazide (IIb)

(c) β-Aspartyl or γ-glutamyl residue in peptide or carboxyl-terminal asparagine or glutamine,

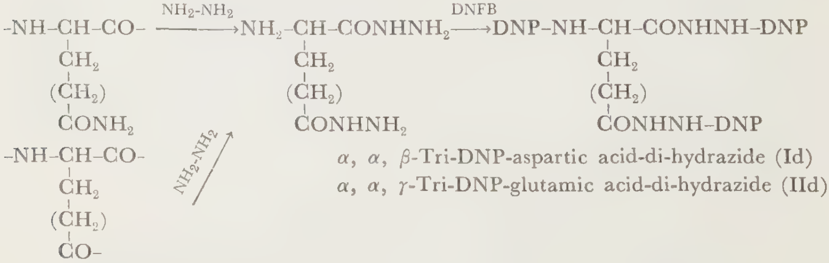


$$\text{-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CO-}}{CH}\text{-COOH}\xrightarrow{NH_2\text{-}NH_2}NH_2\text{-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CONHNH_2}}{CH}\text{-COOH}\xrightarrow{DNFB}\text{DNP-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CONHNH\text{-}DNP}}{CH}\text{-COOH}$$

$$\text{-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CONH_2}}{CH}\text{-COOH}\xrightarrow{NH_2\text{-}NH_2}$$

α, β-Di-DNP-aspartic acid-β-hydrazide (Ic)
α, γ-Di-DNP-glutamic acid-γ-hydrazide (IIc)

(d) Asparaginyl or glutaminyl residue in peptide, or branching at their two carboxyl groups,



$$\text{-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CONH_2}}{CH}\text{-CO-}\xrightarrow{NH_2\text{-}NH_2}NH_2\text{-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CONHNH_2}}{CH}\text{-CONHNH}_2\xrightarrow{DNFB}\text{DNP-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CONHNH\text{-}DNP}}{CH}\text{-CONHNH-DNP}$$

$$\text{-NH-}\underset{\substack{|\\ CH_2\\ |\\ (CH_2)\\ |\\ CO-}}{CH}\text{-CO-}\xrightarrow{NH_2\text{-}NH_2}$$

α, α, β-Tri-DNP-aspartic acid-di-hydrazide (Id)
α, α, γ-Tri-DNP-glutamic acid-di-hydrazide (IId)

By the fractional extractions described in the previous paper (*3*), Ia, Ib, Ic, IIa, IIb, and IIc can be extracted to acidic fraction owing to their free carboxyl groups, while Id and IId remain in nonacidic fraction. The former six derivatives can be separated on silica gel chromatograms (*4*), and, Ib, Ic, IIb, and IIc can be estimated colorimetrically as DNP-aspartic or glutamic acid after hydrolysis of the hydrazide bond with hydrochloric acid. Id and IId can also be estimated colorimetrically after hydrolysis and separation on chromatogram from many other DNP-amino acid hydrazides (which have been also converted to DNP-amino acids). Thus, four types of linkage of acidic amino acids can be determined quantitatively.

The author has applied this technique to lysozyme and obtained a result which indicates that lysozyme has sole α-aspartyl residue in the peptide, and the other 12 aspartic and 4 glutamic acids exist as amides.

EXPERIMENTAL

Anhydrous Hydrazine—Anhydrous hydrazine was prepared by distilling 80 per cent hydrazine hydrate with calcium oxide according to Stähelr (*5*). This preparation gave the purest reagent (99–100 per cent). In fact, with this anhydrous hydrazine we have got better results than those reported in the previous paper ; namely, the recovery of lecucine after 10 hours' " hydrazinolysis " was about 90 per cent, and no other byproducts were found. From the hydrazinolysate of lysozyme, leucine was recovered 0.9 moles per mole of the protein assuming the molecular weight to be 15,000.

Hydrazinolysis, dinitrophenylation and Fractionation—Isoelectric lysozyme (20.114 mg., 1.34μM) was hydrazinolysed with about 1 g. of anhydrous hydrazine for 10 hours at 100°. The reaction mixture was evaporated *in vacuo* over sulfuric acid to remove excess hydrazine. The procedure of dinitrophenylation was somewhat modified to minimize any possible hydrolysis of hydrazides during the dinitrophenylation. The residue was dissolved in 10 ml. of water containing 0.2 g. of sodium bicarbonate, added 20 ml. of ethanol solution containing 0.2 ml. of dinitrofluorobenzene, and shaken for 2 hours. After adding 30 ml. of water to the reaction mixture, it was acidified with 2 N hydrochloric acid and extracted with 2×30, 2×20, and 10 ml. of ethyl acetate in which all the DNP derivatives of amino acid and hydrazides were extracted. The ethyl acetate extracts were combined and extracted with 100, 2×60, and 3×30 ml. of 2 per cent sodium bicarbonate solution. By this treatment DNP-amino acid (carboxyl-terminal, leucine) and di-DNP-acidic amino acid mono-hydrazide (in the case of lysozyme, di-DNP-aspartic acid-α-hydrazide only) were extracted by the sodium bicarbonate solution (acidic fraction), while the other DNP derivatives of amino acid hydrazides remained in the ethyl acetate (ethyl acetate fraction).

Treatment of Acidic Fraction—This fraction was washed once with ethyl acetate, and the latter was, after being re-extracted with sodium bicarbonate solution, combined with the main ethyl acetate fraction. The bicarbonate fraction, combined with washing, was acidified with 2 N hydrochloric acid and re-extracted with 50, 30, and 20 ml. of ethyl acetate. The final ethyl acetate solution containing acidic substances was evaporated *in vacuo*, and the residue was hydrolysed with 6 N hydrochloric acid for 5 hours, whereby di-DNP-acidic amino acid hydrazide was converted to DNP-amino acid. The hydrolysate was then extracted with ethyl acetate and divided into equal six parts. When, after evaporating *in vacuo*, each part corresponding to 3.352 mg. (0.224 μM) of lysozyme was chromatographed on sllica gel, only DNP-leucine and DNP-aspartic acid were detected. These DNP-amino acids were cut and estimated colorimetrically, whereby 54.2 γ (0.200 μM) of DNP-leucine and 59.1 γ (0.198 μM) of DNP-aspartic acid were found. By another experiment which was the same as reported previously (*3*), it has been confirmed qualitatively that certain aspartic acid residue in lysozyme appeared as di-DNP-aspartic acid-α-hydrazide, but not as β-hydrazide, in the course of the treatment. Considering some decomposition and loss during the treatment, it might be possible to conclude that lysozyme has one α-aspartyl residue and no glutamyl residue in its peptide chain.

Treatment of the Ethyl Acetate Fraction—To confirm the conclusion described above, the contents of aspartic and glutamic acids in the ethyl acetate fraction, in which these amino acids were present as tri-DNP-amino acid-dihydrazides, were estimated. To do this, the ethyl acetatate fraction was evaporated *in vacuo*, hydrolysed with 6 *N* hydrochloric acid for 5 hours, extracted with ethyl acetate, divided into equal 50 parts, and each part was evaporated *in vacuo*. Each of these parts was chromatographed, and the bands corresponding to DNP-aspartic and glutamic acids, and further, for references, DNP-serine and threonine were cut, and these were estimated colorimetically, whereby 72.5 γ (0.250 μM) of DNP-aspartic acid, 25.6 γ (0.082 μM) of DNP-glutamic acid, 54.5 γ (0.201 μM) of DNP-serine, and 45.5 γ (0.159 μM) of DNP-threonine were found from one portion corresponding to 0.4023 mg. (0.0265 μM) of lysozyme.

TABLE I

Analysis of Certain Amino Acid Residues in Lysozyme(a)

Amino acid residue	Type of linkage in peptide	Number of residue per molecule		Recovery factor(c)	References	
		Found	Corrected(b)		Fromageot *et al.* (*6*)	Lewis *et al.* (*7*)
Leucine	C-terminal	0.90	1	0.90		
Aspartic acid	α-Aspartyl	0.88	1	0.88	13	20
Asparagine	Asparaginyl	9.33	12	0.78		
Glutamine	Glutaminyl	3.10	4	0.78	4	4
Serine	Seryl	7.95	10	0.80	10	10
Threonine	Threonyl	5.92	7	0.84	7	7
Amide-N					16	18
Free COOH belonging to acidic amino acids					1	6

(a) Isoelectric lysozyme analyzed is 20.114 mg. (1.34 μM).

(b) Possible integral number of residue considering the recoveries of each amino acids are nearly the same as that of leucine.

(c) Computed from the data of the 3rd and 4th columns.

Although no control experiments to estimate the recovery of amino acids in the course of hydrazinolysis have been done, it might be possible to conclude from the data obtained here that lysozyme has one α-aspartyl, 12 asparaginyl, and 4 glutaminyl residues in its peptide chain. The results are cited in Table I.

DISCUSSION

As described already, the tri-DNP-acidic amino acid-di-hydrazides are derived not only from their amide type but also from the branch type of these amino acids, but, from the analytical data on amide nitrogen and from the fact that lysozyme consists of one polypeptide chain, 12 aspartic and 4 glutamic acid residues must exist as amides.

In lysozyme only one aspartic acid exists as its carboxyl-free in the peptide chain, and, therefore, di-DNP-aspartic acid-α-hydrazide is the sole acidic amino acid derivative appeared in the acidic fraction. But the other three DNP-derivatives of acidic amino acid mono-hydrazides, if exist, could be characterized and estimated in analogous way.

Although we have not tried to estimate other amino acid hydrazides than those cited in Table I, recoveries of these amino acid hydrazides were seemed to agree fairly well with those reported by Fromageot *et al.* (*6*), provided that the decomposition of these amino acids during hydrazinolysis is nearly equal to that of leucine and some losses are permitted owing to their many-step treatment.

Lewis *et al.* (*7*) reported that lysozyme has 20 aspartyl and 4 glutamyl residues and 18 amide nitrogen per molecule. Computing from their data, lysozyme must have 6 free carboxyl groups other than carboxyl-terminal. This could not be acceptable from our results.

The fact that no amino acid derivatives other than leucine and aspartic acid appeared in the acidic fraction indicates that DNP-derivatives having carboxyl group can be separated perfectly from other DNP-hydrazides.

It must be emphasized that one must take care to carry out the dinitrophenylation and the separation treatment as fast as possible (within about 5 hours), because some hydrolysis of hydrazide bonds into amino acids might occur by prolonged contact with dilute acid or alkali.

SUMMARY

1. A method for characterization of aspartyl, asparaginyl, glutamyl and glutaminyl residues in peptide chains is described.

2. Lysozyme is proved to possess one α-aspartyl residue, and no

glutamyl residue in its peptide chain. Other aspartic and glutamic acids exist as amides in it.

The author wishes to express his gratitude to Professor S. Akabori for his kind guidance and encouragements through this investigation, to Mr. Haruna for his technical assistance.

REFERENCES

(*1*) Jollès, P., and Fromageot, C., *Biochim. et Biophys. Acta*, **9**, 287, 416 (1952)
(*2*) Chibnall, A. C., and Rees, M. W., *Biochem. J.*, **52**, iii (1952)
(*3*) Ohno, K., *J. Biochem.*, **40**, 621 (1953)
(*4*) Akabori, S., Ohno, K., Nagata, A., and Haruna, I., The 6th Ann. Meeting of Chem. Soc. Japan (Kyoto, April 1953)
(*5*) Stähler, A., *Ber. dtsch. chem. Ges.*, **42**, 3018 (1909)
(*6*) Fromageot, C., and Privat de Garilhe, M., *Biochim. et Biophys. Acta*, **4**, 509 (1950)
(*7*) Lewis, J. C., Snell, N. S., Hirshman, D. J., and Fraenkel-Conrat, H., *J. Biol. Chem.*, **186**, 23 (1950)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

KINETIC STUDIES ON THE ACTION OF GLUCOSE DEHYDROGENASE

IV. QUANTITATIVE ANALYSIS OF THE INHIBITORY ACTION OF URETHANE

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(Received for publication, December 22, 1953)

In previous papers of this series (1–6) the mode of action displayed by the glucose dehydrogenase from *Aspergillus oryzae* has been systematically studied from kinetic point of view. In continuation of this study we investigated the mechanism of inhibitory action of urethane (ethyl carbamate) upon the enzyme. It was found that the narcotic inhibits the enzymatic reaction by combining with the enzyme molecule without competing with the substrate or with the hydrogen-acceptor. The experimental data and theoretical considerations bearing on this subject are presented herewith.

METHODS

Preparation of the enzyme and the measurement of its activity (using thionine, 2,6-dichlorophenol-indophenol or *p*-quinone as hydrogen-acceptor, and D-glucose or other sugars as substrate) were performed as previously described (*2*).

RESULTS

Reversibility of Inhibition—To test whether urethane reacts reversibly or irreversibly with the enzyme molecule, the following experiment was carried out. One half ml. of buffered enzyme solution was previously mixed with 0.5 ml. urethane solution (1.0 *mole/lit.*). After 0, 10, 30 and 60 minutes, the reaction was initiated by introducing 0.5 ml. of 2,6-dichlorophenol-indophenol ($2\times10^{-3{\cdot}0}$ *mole/lit.*) and 0.5 ml. of D-glucose solution (0.6 *mole/lit.*). It was found that the rate of reaction was quite independent of the duration of pretreatment by urethane, the observed rate of reaction being in all cases $5.7\times10^{-5{\cdot}0}\ [\frac{mole}{lit.}]\ /\ [\frac{g.}{lit.}]\ [min.]$

From this result it may be concluded that the reaction, in which urethane combines with enzyme molecule, is not only very rapid, but also reversible.

Relation between the Degree of Inhibition Caused by Urethane and the Concentration of Hydrogen Acceptor—Using 2,6-dichlorophenol-indophenol as hydrogen acceptor, the effect of urethane in a definite concentration (0.25 *mole/lit.*) was compared at different concentrations of the hydrogen acceptor. The results obtained are summarized in Table I. The degree of inhibition (H) was calculated by

$$H=1-\frac{V_u}{V_o}$$

Table I

Effect of Initial Concentration of Hydrogen-acceptor on the Rate of Reaction in the Presence of Urethane

Buffered enzyme solution (2 mg./ml.), 0.5 ml.; 2.6-dichlorophenol-indophenol (2×10^{-3} *mole/lit.*), 0.8 ml., 0.6 ml., 0.4 ml. or 0.2 ml.; D-glucose solution (2.5 *mole/lit.* or 0.6 *mole/lit.*), 0.2 ml.; urethane solution (1.0 *mole/lit.*), 0.5 ml.

Total volume of reaction mixture was made up to 2.0 ml. with distilled water. Temperature: 30°; pH: 7.2.

(A). The concentration of D-glucose was 0.25 *mole/lit.* The rate in the absence of urethane was 12.4×10^{-5} $[\frac{mole}{lit.}]/[\frac{g.}{lit.}]$ [*min.*]

Initial concentration of hydrogen-acceptor ([*mole/lit.*])	Rate of reaction in the presence of 0.25 *mole/lit.* urethane ($[\frac{mole}{lit.}]/[\frac{g.}{lit.}]$ [*min.*])	Degree of inhibition (H)
0.8×10^{-3}	$6.1_0\times10^{-5\cdot0}$	0.51
0.6×10^{-3}	$6.2_5\times10^{-5\cdot0}$	0.49
0.4×10^{-3}	$6.2_5\times10^{-5\cdot0}$	0.50
0.2×10^{-3}	$6.2_5\times10^{-5\cdot0}$	0.49

(B). The concentration of D-glucose was 0.06 *mole/lit.* The rate in the absence of urethane was $9.9_5\times^{-5}$ $[\frac{mole}{lit.}]/[\frac{g.}{lit.}]$ [*min.*]

Initial concentration of hydrogen-acceptor ([*mole/lit.*])	Rate of reaction in the presence of 0.25 *mole/lit.* urethane ($[\frac{mole}{lit.}]/[\frac{g.}{lit.}]$ [*min.*])	Degree of inhibition (H)
0.8×10^{-3}	$5.1_5\times10^{-5\cdot0}$	0.48
0.6×10^{-3}	$5.2_0\times10^{-5\cdot0}$	0.48
0.4×10^{-3}	$5.2_0\times10^{-5\cdot0}$	0.48
0.2×10^{-3}	$5.2_0\times10^{-5\cdot0}$	0.48

where V_u and V_0 represent the rate of enzymatic reaction in the presence and absence, respectively, of urethane. It may be seen from the table that the rate as well as the degree of inhibition was independent of the initial concentration of hydrogen-acceptor. Also in the presence of urethane, the reaction was found to be of zero order in respect to the concentration of hydrogen-acceptor up to 10^{-4} *mole/lit.* This result indicates that urethane does not compete with the hydrogen-acceptor for the enzyme molecule.

Degree of Inhibition Observed in the Presence of Different Hydrogen Acceptors—By plotting the degree of inhibition against the logarithms of concentration of urethane ($pU = -\log_{10} U$), we obtained, as illustrated in Fig. 1, a sigmoid curve of the first order, which corresponds to the equation:

$$H = \frac{[U]}{\phi + [U]}$$

where [U] is the concentration of urethane and ϕ a characteristic constant corresponding to [U] causing 50 per cent inhibition.

Using various hydrogen acceptors, the H-pU-relationship was investigated at a given concentration of D-glucose (see Fig. 1). As may be seen from Table II, the ϕ-value obtained was the same for different hydrogen acceptors applied.

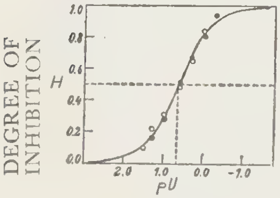

FIG. 1. The inhibition-pU-curve in the presence of 0.1 *mole/lit.* D-Glucose. (Temperature: 30°, pH: 7.2).

○ : Observed value, using 2,6-dichlorophenol-indophenol as hydrogen-acceptor.

● : Observed value, using quinone as hydrogen-acceptor.

TABLE II

The Value of ϕ Found in the Presence of Different Hydrogen-acceptors

The concentratson of D-glucose was 0.10 *mole/lit.*
(Temperature: 30°; pH: 7.2)

Hydrogen-acceptor	Thionine	2,6-Dichlorophenol-indophenol	*p*-Quinone
value of [*mole/lit.*]	0.23	0.25	0.25

Degree of Inhibition Observed in the Presence of Different Substrates in Different Concentrations—From the results summarized in Fig. 2 and Table III, it is apparent that the value ϕ (0.25 *mole/lit.* at 30°) was not affected by the concentration of the substrate. The comparison of the ϕ-values using other substrates of the enzyme, *i.e.* D-galactose,

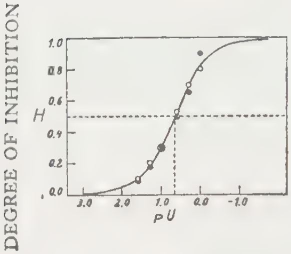

FIG. 2. The inhibition-pU-curve at various concentrations of D-Glucose.

Hydrogen-acceptor: thionine. (Temperature: 30°; pH: 7.2)

○: Observed value in presence of 0.1 *mole/lit.* D-glucose.

●: Observed value in presence of 0.0083 *mole/lit.* D-glucose.

TABLE III

Effect of the Concentration of Substrate upon the Value of ϕ

(A) Hydrogen-acceptor: 2,6-dichlorophenol-indophenol

Concentration of D-glucose	0.10 *mole/lit.*	0.05 *mole/lit.*	0.0125 *mole/lit.*
Value of ϕ [*mole/lit.*]	0.25	0.25	0.25

(B) Hydrogen-acceptor: thionine

Concentration of D-glucose	0.10 *mole/lit.*	0.05 *mole/lit.*	0.0125 *mole/lit.*
Value of ϕ [*mole/lit.*]	0.25	0.25	0.25

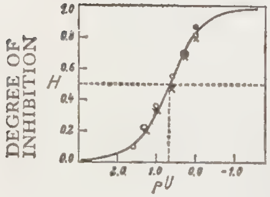

FIG. 3. The inhibition-pU-curve, when D-galactose, D-mannose and D-xylose were used as substrate.

Hydrogen-acceptor: 2,6-dichlorophenol-indophenol. (Temperature: 30°; pH 7.2).

○: Observed value in presence of 0.1 *mole/lit.* D-glactose.

●: Observed value in presence of 0.5 *mole/lit.* D-mannose.

×: Observed value in presence of 0.15 *mole/lit.* D-xylose.

D-mannose and D-xylose, showed that it is also entirely independent of the nature of substrates (see Fig. 3).

Effect of Temperature—The effect of temperature on the ϕ-value of urethane was investigated using D-glucose (0.15 *mole/lit.*) as substrate. The results listed in Table

IV show that the ϕ-value remained unchanged in the temperature range between 0° and 30°.

TABLE IV

Effect of Temperature upon the Value of ϕ

Buffered enzyme solution (0.125 *mole/lit.* phosphate), 0.5 ml.; 2,6-dichlorophenol-indophenol ($2\times10^{-3.0}$ *mole/lit.*), 0.5 ml.; D-glucose solution (0.60 *mole/lit.*), 0.5 ml.; urethane solution or distilled water, 0.5 ml.

Total volume: 2.0 ml. pH: 7.2

Temperature	303°K	291°K	282°K	273°K
Value of ϕ [*mole/lit.*]	0.25	0.25	0.27	0.27

DISCUSSION

As shown in previous papers, the mechanism of the action of glucose dehydrogenase may be represented by the following sequence of reactions;

$$\mathrm{E+F} \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} \mathrm{EF} \qquad (1),$$

$$\mathrm{EF+S} \underset{k_2'}{\overset{k_2}{\rightleftharpoons}} \mathrm{EFS} \qquad (2),$$

$$\mathrm{EFS} \xrightarrow{k_3} \mathrm{E+H_2F+P} \qquad (3),$$

where E is the free enzyme molecule, F the hydrogen acceptor, S the substrate, P the product derived from the substrate, and EF and EFS the complexes formed of the molecules indicated. k_1, k_2 and k_3 are the velocity constants for the reactions from left to right, and k'_1 and k_2' for the reactions from right to left.

Based on the fact that the H-pU-relationship represents a sigmoid curve of the first order, we may infer that one urethane molecule combines with one enzyme molecule. On the other hand, we have seen that urethane competes neither with substrate nor with hydrogen acceptor in their reaction with enzyme molecule. It may, therefore, be reasonable to assume that urethane (U) combines not only with E, but also with EF and EFS; namely:

$$\mathrm{E+U} \overset{\mathrm{K_U}}{\rightleftharpoons} \mathrm{EU} \qquad (4),$$

$$\mathrm{EF+U} \overset{\mathrm{K_U}}{\rightleftharpoons} \mathrm{EFU} \qquad (5),$$

$$\mathrm{EFS+U} \overset{\mathrm{K_U}}{\rightleftharpoons} \mathrm{EFSU} \qquad (6),$$

where Ku represents the dissociation constant of the urethane-enzyme complexes.

Stationary state kinetics based on the Eqs. (1)–(6) leads to the following equation:

$$\mathrm{Vu}=\frac{k_3 t}{(1+\frac{[\mathrm{U}]}{\mathrm{K_U}})\ \{\frac{k_1'(k_2'+k_3)+k_2k_3[\mathrm{S}]}{k_1k_2[\mathrm{S}][\mathrm{F}]}+\frac{k_2'+k_3}{k_2[\mathrm{S}]}+1\}}$$

where $\varepsilon=\mathrm{E+EF+EFS+EU+EFU+EFSU}$.

If, as has been shown previously (*cf. 2*), the first term in the braces of denominator may be neglected as a small term, we have

$$\mathrm{Vu}=\frac{k_3 t}{(1+\frac{[\mathrm{U}]}{\mathrm{K_U}})\ \{\frac{k_2'+k_3}{k_2[\mathrm{S}]}+1\}} \qquad (7),$$

and

$$\mathrm{H}=\frac{[\mathrm{U}]}{\mathrm{K_U}+[\mathrm{U}]} \qquad (8).$$

This equation shows that the ϕ-value experimentally obtained represents the dissociation constant of urethane-enzyme-complexes. Using the data given in Table IV, thermodynamical quantities of the forward reaction between urethane and enzyme molecule was estimated. The values obtained are as follows:

$$\varDelta \mathrm{F}(303^\circ\mathrm{K})=-833\ [\frac{cal.}{mole}]$$

$$\varDelta \mathrm{S} = 2.75\ [\frac{cal.}{mole\cdot deg.}]$$

$$\varDelta \mathrm{H} = 0\ [\frac{Kcal.}{mole}]$$

SUMMARY

1. The inhibitory action of urethane upon the reaction catalyzed by the glucose dehydrogenase from *Aspergillus oryzae* was investiaged from kinetic point of view.

2. The inhibition caused by urethane is reversible. The narcotic combines with the enzyme without competing with hydrogen-acceptor

and substrate. It is, therefore, assumed that urethane combines not only with free enzyme molecule but also with enzyme-acceptor-complex as well as with enzyme-acceptor-substrate-complex, and indeed, with the same dissociation constant in all cases. Experimental evidence was adduced which indicates that the dissociation constant mentioned above remains the same (0.25 *mole/lit.*) for different hydrogen acceptors and different substrates. It is also independent of temperature in the range from 0° to 30°.

3. Based on the data obtained, thermodynamical quantities of the reaction in which urethane combines with the enzyme molecule was estimated.

The author wishes to thank Prof. Hiroshi Tamiya for his valuable suggestions and encouragement in this work.

REFERENCES

(*1*) Ogura, Y., *Acta Phytochim.*, **11**, 127 (1939)
(*2*) Nagahisa, M., and Ogura, Y., *Bot. Mag.* (*Tokyo*), **56**, 137 (1942)
(*3*) Ogura, Y., *J. Biochem.*, **38**, 75 (1951)
(*4*) Ogura, Y., *J. Biochem.*, **39**, 31 (1952)
(*5*) Ogura, Y., *J. Biochem.*, **39**, 287 (1952)
(*6*) Ogura, Y., *J. Biochem.*, **39**, 311 (1952)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

II. EFFECT OF BENZOATE UPON THE AUTOXIDATION OF OXYHEMOGLOBIN

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(Received for publication, December 22, 1953)

The essential function of hemoglobin as an oxygen transporting system can easily be affected even through a mild structural alteration of its globin part, for instance, through a reversible denaturation caused by acids, by pyridine or by addition of Cu^{++}. Interestingly enough, this is accompanied by an alteration of its catalytic function, the modified hemoglobin thus becomes significantly easily autoxidizable as painted out by Holden (*1*, *2*), Rawlinson (*3*) and Lemberg (*4*).

In his previous paper, the present author reported on the spectral changes of hemoglobin and hemiglobin induced by the addition of benzoate. These spectral changes were suggested by the author to be the result of the coordinative linking of heme-Fe to another globin-N group which was opened out from the globin molecule through its configurational modification induced by benzoate. From his interest on the interrelation between the structure of hemoglobin and its catalytic function, the present author intended to study the possible alteration of the catalytic function of oxyhemoglobin according to the structural modification of its globin part by benzoate.

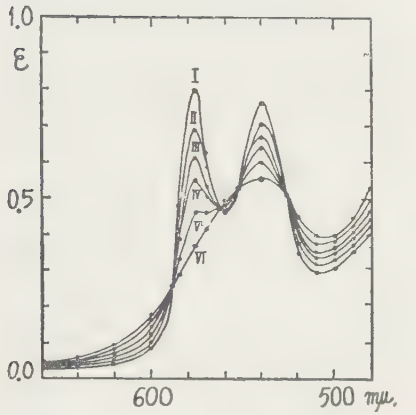
EXPERIMENTAL METHODS

Bovine erythrocytes repeatedly washed by physiological saline solution were laked by addition of distilled water and centrifuged. The supernatant was used as the oxyhemoglobin solution for the present experiments. All the blood samples were reserved in ice box and used for the experiments within 24 hours. Concentrations of oxyhemoglobin were checked at each experiment by the determination of its optical density at 578 mμ. 2 *M* solution of benzoate was prepared as the stock solution, from which the solutions of desired concentrations were made each time by dilution.

Each of the solutions of oxyhemoglobin and benzoate was separately pipetted into beakers of 200 ml. content and incubated at 30°. Two solutions were then mixed after they reached the constant temperature. 9.5 ml. of the reaction solution was pipetted out from the beaker at certain intervals of time, 0.5 ml. of 1.0 per cent KCN solution was added and the spectrophotometric reading was made within 1 minute. pH of the solution was determined by means of glass electrode. Temperature of the experiments was kept constant at 30°, except those of the temperature change. The optical determinations were carried out by the photoelectric spectrophotometer of Hitachi Company.

RESULTS AND INTERPRETATIONS

I. Determination of the Reaction Rate by Absorptiometrical Observations—To the solution of oxyhemoglobin, benzoate was added to its final concentration of 1.785 *M*. Optical density was measured after 30 minutes of incubation at 30°. An absorption figure thus recorded was perfectly similar to that obtained from the solution of methemoglobin after the reaction with benzoate (*5*) *i.e.* a typical hemichrome figure. Thus, for the present reaction process, two possible modes of reaction may be taken into consideration, one of which being the way to hemichrome from oxyhemoglobin directly and the other being the way to hemichrome stepwise from oxyhemoglobin through methemoglobin as an intermediate. When, however, KCN was added to this hemichrome like compound, an absorption figure was obtained which was similar to CN-methemoglobin. The process of the spectral change of oxyhemoglobin into hemichrome was thus followed up, for convenience, by the CN-methemoglobin spectrum formed on addition of KCN into the reaction solution. The problem regarding the reaction modes will be discussed in a later section. To the solution of oxyhemoglobin, benzoate was added to its final concentration of 1.052 *M* and after certain intervals of time, KCN was added to it. The resulted absorption figure was thus recorded as in Fig. 1. The absorption maxima at 578 and 540 mμ decreased with time until finally the figure of CN-methemoglobin was formed. The curves in Fig. 1 make four isosbestic points indicating the reaction system of two components: oxyhemoglobin and CN-methemoglobin, the latter being formed from hemichrome by KCN added. The percentage of oxyhemoglobin autoxidized in the system could then be obtained from the value of ε_{578} by a nomogram established by a procedure similar to that described in the previous paper (*5*). Now the percentages of autoxidation were determined at varied concentrations of benzoate and after each time interval. The plots of log percentages of remaining oxyhemoglobin against time were recorded in Fig. 2. The relation was



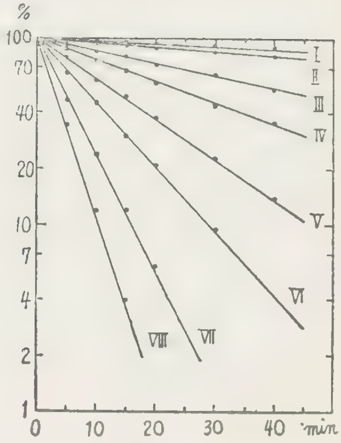


FIG. 1. The absorption change on addition of benzoate to oxyhemoglobin solution.

On measurements, KCN solution was added, so that the final figure recorded corresponds to that of CN-methemoglobin. Final concentration of benzoate was 1.052 *M*. The readings were made after the following intervals of time.

I; 0 min. II; 10 min. III; 20 min. IV; 30 min.
V; 50 min. VI; 120 min. pH=7.2 T=30°

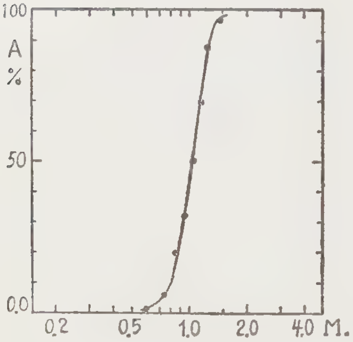
FIG. 2. The plots of log percentages of remaining oxyhemoglobin against time.

Concentration of oxyhemoglobin; 0.085% Concentration of benzoate; I; 0.61 *M*, II; 0.74 *M*, III; 0.86 *M*, IV; 0.965 *M*, V; 1.052 *M*, VI; 1.17 *M*, VII; 1.26 *M*, VIII; 1.42 *M*, pH; 7.2, T=30°

given as a straight line at each of the benzoate concentrations, indicating a first order reaction in respect to the concentration of oxyhemoglobin. Now, taking V_O as the reaction velocity without benzoate and V_B as that with benzoate, the percentage of increase in the reaction velocity (A) promoted by benzoate can then be expressed by $A=(1-\frac{V_B}{V_O})\times 100$. From experiments with benzoate in varied concentrations, the percentages of remaining oxyhemoglobin after 15 minutes were determined. By introducing these values into V_O and V_B of the above equation, the value of A was calculated. As shown in Fig. 3, the plots of A against log concentrations of benzoate gave a sigmoid curve. The value of n was calculated from the curve to be about 4.5. From this value, it may be assumed that the autoxidation of oxyhemoglobin seems to begin with the combination of globin molecule with 4 to 5 molecules of benzoate. Clarification of the actual feature of the combination between these two components, must await further experimentation.

II. Effect of Temperature—If the reaction by benzoate described above is to be assumed as the result of perturbation occuring in the molecule of protein part, it may be expected that the reaction can greatly be affected by temperature. The following experiments were undertaken, therefore, to investigate this point.

As shown in Fig. 4, the reaction rate increased with increase of temperature, the final concentration of benzoate being kept constant at 1.58 *M.* The rate constants k were thus obtained at each temperature. The plots of log k against the reciprocals of absolute temperature



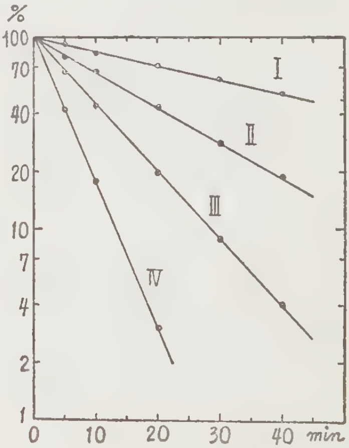


Fig. 3. The plots of A (percentage of velocity increase) against log concentrations of benzoate.

Fig. 4. The effect of temperature upon the reaction velocity.

Concentration of oxyhemoglobin; 0.085% Concentration of benzoate; 1.30 *M.* pH; 7.2 Temperature; I 16°; II 22°; III 26°; IV 30°

revealed a linear relationship between them (Fig. 5). From the slope of the line the activation energy of Arrhenius was calculated to be 28.7 Cal./mole. The energy value thus obtained should be understood as the sum consisting of the energy of the combination of globin and benzoate, that of the structural change within protein molecule and that of the activation of molecular oxygen. Though the energy value obtained thus cannot be exclusively accounted for by the reaction of combination step of globin and benzoate, it is very much smaller than those so far reported for the reaction of protein denaturation, for instance, for the reactions of heat denaturation or alcohol denaturation of oxyhemoglobin (*6*, *7*). It may then be assumed, at least, that the affection of benzoate upon oxyhemoglobin (by which the latter becomes easily autoxidizable) may be far from a sort of denaturation in the classical meaning but a slight structural modification of its globin part.

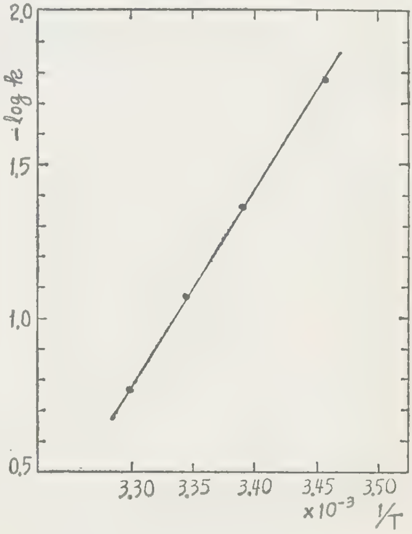
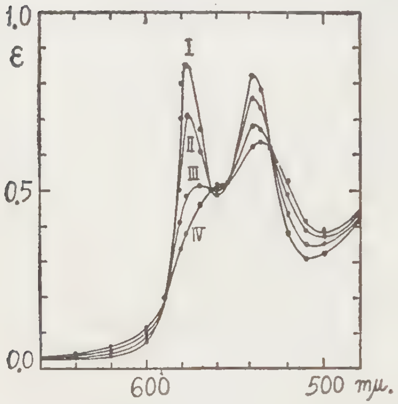


Fig. 5 The plots of log k against the reciprocals of absolute temperature.

II. Reaction Process of the Autoxidation—Two possible elucidations for the above mentioned reaction process present themselves: a) If the autoxidation of oxyhemoglobin occurs only after its protein part was so much affected by benzoate that the resulting heme-protein gives a hemichrome figure in its Fe^{+++} state, then the presented reaction process may correspond simply to a conversion of oxyhemoglobin to hemichrome; b) but, if the structural modification of oxyhemoglobin necessary for inducing its autoxidation is slighter, then an intermediary formation of methemoglobin may be suggested and the latter may immediately turn into hemichrome. In order to explain the presented reaction, the following experiments were undertaken. Benzoate was added to oxyhemoglobin solution and the absorption changes thus occured were followed up at certain time intervals. Results are shown in Fig. 6.

including isosbestic points in the curves. From this the possibility of intermediary formation of methemoglobin can apparently be excluded. At this benzoate concentration, however, the conversion from methemoglobin to hemichrome took place almost instantly as reported in the previous paper, so that the intermediary methemoglobin, even though it might have been actually formed, could not be detected with certainty. The problem remains as yet a matter of conjecture.

IV. Accessibility of Several Heme-proteins to Benzoate—From the results of the present experiments including that of the spectral changes reported in the previous paper, the accessibility of several proteins to benzoate are summarized in Fig. 7. As may be seen from this figure, the



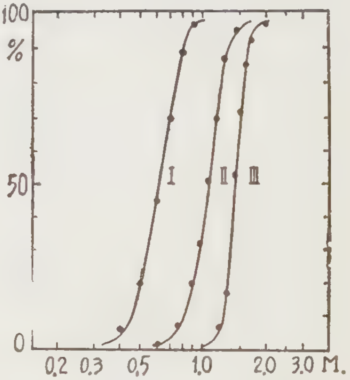


Fig. 6. The absorption change of oxyhemoglobin to hemichrome by the addition of benzoate to its final concentration of 1.052 *M*.

The resulted absorption figures were obtained at the following intervals of time: I 0 min.; II 10 min.; III 45 min.; IV 120 min.; pH 7.2 T=30°.

Fig. 7. The accessibility of methemoglobin, oxyhemoglobin and reduced hemoglobin to benzoate. (Summarized from the experimental results described above and reported in the previous paper).

I : from the absorption change of methemoglobin to hemichrome,
II : from the autoxidation velocity of oxyhemoglobin,
III : from the absorption change of reduced hemoglobin to hemochrome.

protein parts of heme-proteins, methemoglobin, oxyhemoglobin and reduced hemoglobin, were affected by benzoate in progressively higher concentrations, in the given order. The accessibility of globin molecule to benzoate seems to be dependent on its molecular state in each heme-protein. An analogous assumption was afforded by Ross and Turner (*8*), who have found that the stability of heme-proteins against tryptic digestion was greater in the order of methemoglobin, oxyhemoglobin and reduced hemoglobin. Both findings might possibly be explained on the basis of some structural reasons in common.

On the other hand, it was suggested by Perutz *et al.* (*9*) from their X-ray crystallographic studies, that the globin part in the oxyhemoglobin molecule is similar to that in the molecule of methemoglobin, and that it differs from that in reduced hemoglobin. Further, Wyman (*10*) suggested the structural similarity of oxyhemoglobin to methemoglobin on the basis of experimental results indicating that the Bohr effect observed in the oxidative conversion of hemoglobin to methemoglobin is essentially the same as that found in the oxygenation of hemoglobin and also in connection with the results of X-ray study by Perutz *et al.* (*9*).

The concentration of benzoate that is effective to induce the autoxidation of oxyhemoglobin was found in the present experiment, as described above, to be much higher than that effective to induce the spectral conversion of methemoglobin into hemichrome. From these findings connected with the above mentioned assumption of Wyman (*10*), it would not be unreasonable at present to assume that the protein part of oxyhemoglobin may have been more stabilized through its combination with O_2 than that of methemoglobin. The suggestion remains as yet to be proved with evidence; how far the autoxidation of oxyhemoglobin including the process of O_2 activation can be connected with the structural modification of globin in the chemical meaning as well as in the grade of the change remains so far unknown. The structural similarity between methemoglobin and oxyhemoglobin was proposed by Wyman (*10*) on the experimental basis of Bohr effect. But, how far the Bohr effect is in accord with the structural state of the globin part remains also to be investigated. From the assumption that reduced hemoglobin is in random structure, as suggested by Wyman (*10*), and from the fact that reduced hemoglobin can be affected only by higher benzoate concentrations than methemoglobin, it may further be suggested that the stability of heme-proteins against perturbators is highly dependent on the physical state of heme part in each of their molecules.

SUMMARY

1. Benzoate accelerated the autoxidation of oxyhemoglobin.

2. The autoxidation of oxyhemoglobin in the presence of benzoate proceeded according to the first order kinetics in respect to the concentration of oxyhemoglobin.

3. The relation between the percentage promotion of the autoxidation of oxyhemoglobin and the log concentration of benzoate could be expressed by a sigmoid curve of a high order reaction.

4. Activation energy in the autoxidation of oxyhemoglobin induced by benzoate was calculated to be 28.7 Cal./mole.

5. The stability of globin molecule against the action of benzoate differs with the difference of the physical state of their heme part.

This study was undertaken at the suggestion by Prof. K. Kaziro to whom the authors deep gratitude is due. I should like to express my thanks also to Dr. G. Kikuchi for his helpful discussion during the course of this work.

REFERENCES

(*1*) Holden, H. F., *Australian J. Exptl. Biol. Med. Sci.*, **14**, 291 (1936)
(*2*) Holden, H. F., *Australian J. Exptl. Biol. Med. Sci.*, **15**, 43 (1937)
(*3*) Rawlinson, W. A., *Australian J. Exptl. Biol. Med. Sci.*, **17**, 53 (1939)
(*4*) Lemberg, R., *Australian J. Exptl. Biol. Med. Sci.*, **20**, 111 (1942)
(*5*) Tsushima, K., *J. Biochem.*, **41**, 215 (1954)
(*6*) Lewis, P. S., *Biochem. J.*, **20**, 965 (1926)
(*7*) Booth, N., *Biochem. J.*, **24**, 1699 (1930)
(*8*) Ross, W. F., and Turner, R., *J. Biol. Chem.*, **139**, 603 (1941)
(*9*) Perutz, M. F., *Proc. Roy. Soc. London. A.*, **195**, 474 (1949)
(*10*) Wyman, Jr., *J. Polymer Sci.*, **7**, 499 (1951)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

EFFECT OF ZINC AND MAGNESIUM IONS IN HIGH CONCENTRATION ON SERUM ALKALINE PHOSPHATASE WITH REFERENCE TO CANCER

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(Received for publication, December 28, 1953)

This communication reports our studies of the competitive effect of zinc and magnesium ions in relatively high concentrations, on the alkaline phosphatase activity of the serum of non-cancerous individuals and of those having a malignant tumor.

Roche (*1*, *2*, *3*) and his associates found that the addition of Zn^{++} in a concentration of 10^{-5} *M* weakly activated the serum alkaline phosphatase activity in non-cancerous individuals, and in contrast, a slight inhibition was observed in the serum of patients with cancer. Bodansky (*4*) found that Zn^{++} 10^{-5} *M* had no effect on the serum alkaline phosphatase activity in individuals either with or without cancer. Similar conclusions were reached by other investigators (*5*, *6*). The activation of alkaline phosphatase activity by magnesium ions has been well established (*7*, *8*, *9*, *10*) and in general magnesium ions are used to reverse the inhibitory effects of other ions, the exception being beryllium (*7*, *11*). We know of no detailed studies on the effect of the addition of both zinc and magnesium ions on the alkaline phosphatase activity of serum. Bodansky (*4*) found that Zn^{++} in a concentration of 0.001 *M* added to serum produced an inhibition of about 50 per cent of serum alkaline phosphatase activity. In our preliminary experiments using 0.001 *M* Zn^{++} as the inhibiting agent, we were able to reverse completely the phosphatase inhibition by the addition of 0.02 *M* Mg^{++}. This is approximately the same ratio of zinc to magnesium found in human serum. The use of higher concentrations of Zn^{++} *viz*: 0.004 *M* resulted in inhibition of alkaline phosphatase activity of about 80 per cent. The reactivation of alkaline phosphatase activity by magnesium ions is limited, since increasing the concentration of Mg^{++} above 0.03 *M* produced no further reversal of the inhibited phosphatase activity. Zinc ions in a concentration of 0.012 M produced total inhibition of alkaline phosphatase activily, and the addition of magnesium ions failed to restore activity.

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* Under a grant from Lewis Memorial Fund.

METHODS

Alkaline phosphatase was determined using adenylic acid as the substrate as previously described (*12*). To the buffered substrate 0.004 M $ZnCl_2$ and 0.03 M $MgCl_2$ were added, and the phosphatase again determined.

RESULTS AND DISCUSSION

Table I records the results of our studies on the effect of the addition of both zinc and magnesium ions on the alkaline phosphatase activity of human serum. Since elevated serum alkaline phosphatase values are generally accepted as indicating either bone disease or liver damage, such cases were excluded from our series.

TABLE I

Statistical Analysis of Effect of Addition of Zinc and Magnesium Ions on Alkaline Phosphatase Activity of Human Serum

(all phosphatase values in mg.%)

Nonmalignant	No. of patient	Control phosphatase	Phosphatase after addition of 0.004 M Zn^{++} and 0.03 M Mg^{++}	Inhibition	Percent inhibition
	82				
Mean		2.2	1.4	0.8	36
Range		0.9–3.8	0.5–3.3	0.1–1.6	3–60
Stand. dev.		0.65	0.51	0.34	11.8
Cancer	56				
Mean		4.7	2.3	2.4	50
Range		2.0–16.4	0.8–11.3	0.9–5.7	33–73
Stand. dev.		2.9	1.7	1.4	7.1
Cancer (with low initial alk. phosphatase)	14				
Mean		2.4	1.2	1.2	50
Range		2.0–2.7	0.8–1.6	1.1–1.6	41–62
Stand. dev.		0.25	0.23	0.29	0.71

The non-cancer group consisted of 22 normal males and females, 19 cases of pregnancy, and 41 others known to be free from neoplastic disease. The cancer group consisted of 56 individuals with some form of neoplastic disease, in whom the diagnosis was confirmed by histological examination. These cases, classified according to the site of the tumor, are as follows: Gastro-intestinal tract 22, genito-urinary system 16, breast 8, lung and esophagus 7, and miscellaneous 3. The phosphatase was determined before surgical intervention or other treatment was instituted.

There is a diminution of the desphorylation of the substrate as a result of the addition of zinc and magnesium ions in both the non-cancerous group, and in those with malignant tumors. The loss of phosphatase activity cannot bo attributed to changes in the pH of the buffer system. The pH of the adenylic acid buffer substarate was 8.90, and after the addition of zinc and magnesium ions was 8.75. In the non-cancerous group, the mean inhibition was 36 per cent, which is equivalent to a mean of 0.8 mg. per cent, while in the cancer group the mean inhibition was 50 per cent, corresponding to a mean of 2.4 mg. per cent. A comparison of the mean of the cancer group with that of the non-cancer group showed a p value, as calculated by the method of Fisher (*13*), of less than 0.01. (When the p value is less than 0.05 it is statistically significant). This applied both to the absolute loss, and in terms of per cent of the original phosphatase activity.

In the 14 cases of cancer with low initial phosphatase activity (Table I), a comparison of the mean of this group with that of the non-cancer group also showed a p value of less than 0.01.

Our results seem to indicate that serum alkaline phosphatase activity which has been inhibited by zinc ions in weak concentrations may be entirely reactivated by the addition of magnesium ions. When the concentration of the zinc ion is markedly increased, the reversal of the inhibition by the addition of magnesium ions is limited.

SUMMARY

The degree of inhibition of the serum alkaline phosphatase activity produced by the addition of relatively high concentrations of zinc and magnesium ions to the buffered substrate seems to be generally higher in the cancer group than in the non-malignant groups.

REFERENCES

(*1*) Roche, J., van Thoai, N., Marcelet, J., and Desruisseaux, G., *Compt. rend. Soc. biol.*, **140**, 632 (1946)

(*2*) Roche, J., Cornil, L., Desruisseaux, G., Baudoin, N., and Long, S., *Compt. rend. Soc. Biol.*, **141**, 1251 (1947)

(*3*) Roche, J., van Thoai, N., Marcelet, J., Desruisseaux, G., and Durnad, S., *Bull. Acad. Med.*, Paris, **130**, 294 (1946)

(*4*) Bodansky, O., and Blumenfeld, O., *Proc. Soc. Ex . Biol. Med.*, **70**, 546 (1949)

(*5*) Fishman, W. H., Wayne, A., and Homburger, F., *Cancer Research*, **9**, 681 (1949)

(*6*) Ellerbrook, L. D., Lippincott, S. W., and Chipps, H. D., *J. Natl. Cancer Inst.*, **11**, 739 (1950)

(*7*) Lehninger, A. L., *Physiol, Rev.*, **30**, 393 (1950)

(*8*) Drill, V. A., Annegers, J. H., and Ivy, A. C., *J. Biol. Chem.*, **152**, 339 (1944)

(*9*) Abul-Fadl, M. A. M., and King, E. J., *Biochem, J.*, **44**, 435 (1949)

(*10*) Bernhard, A., and Rosenbloom, L., *Science*, **118**, 114 (1953)

(*11*) Greier, R. S., Hood, M. B., and Hoagland, M. B., *J. Biol. Chem.*, **180**, 289 (1949)

(*12*) Bernhard, A., and Rosenbloom, L., *Proc. Soc. Exp. Biol. and Med.*, **74**, 164 (1950)

(*13*) Fisher, R. S., Statistical methods for research workers, Edinburgh, 9th Edition, 122 (1944)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

THE STEPWISE NATURE OF PROTEIN DENATURATION

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INTRODUCTION

As one of the important factors for the study of kinetics and thermodynamics of the protein denaturation, the problem about the order of reaction of protein denaturation is frequently discussed and considerable efforts have been made by the pioneers to gain some approach. (*1*).

So far as is known, although this problem is still far from settlement, many observations have already shown the agreement that the protein denaturation obeys the first order law under proper conditions. However, since the denaturation of protein results from the rupture of the secondary bonds between intramolecular polypeptide chains which involve various types of linkage, it seems very unlikely, even from the cursory supposition, that all these bonds are activated and broken down uniformly as if they were unimolecular. Consequently, such an intramolecular change has been supposed to proceed along a smooth first order course in some cases but also to follow a somewhat stepwise course under properly chosen conditions.

In an earlier literature on the protein denaturation, Neurath has already well stated the possibility of this stepwise process. (*1*).

In order to empirically evidence the above conception and Neurath's prophecy, we have tried some investigations as follows:

EXPERIMENTAL

Materials

Horse serum albumin and egg albumin employed were prepared by the modified Keckwick's Method (*2*) and dialyzed in running water for one day and in distilled water for one or two days in an ice-box.

Methods

1. Biuret Reaction—Previously, Araya has found that the weakly alkaline biuret reaction is a sensitive method to observe the mild modification of the intramolecular configuration of proteins (*2*), namely, change in the reactivity of peptide bonds, which would not be revealed by the radical treatment such as the ordinary biuret reaction. Later, he improved this method quantitatively. (*3*). With the advantage of this method, we could observe the gradual change of protein molecules in the course of denaturation.

One per cent solution of horse serum albumin was heated at pH 7 (colorimetrically determined by pH Test Paper B. T. B.) in a thermostat to keep the temperature at 80° or 85° for several hours, of which 3 ml. were pipetted out every 30 minutes from the moment at which the temperature of the solution rose to each temperature required. After these solutions were cooled down to the room temperature (15°) 1 ml. $CuCO_3$ suspension was added. As for the $CuCO_3$ suspension, 1 per cent $CuSO_4 \cdot 5\ H_2O$ and 1 per cent Na_2CO_3 were mixed together and its pH was carefully adjusted prior to mixing with the protein solution so as to bring the initial pH of the protein-suspension mixture to 8.8 (by pH Test Paper T. B.), where the pH itself does not affect the native configuration of the protein. In case of the biuret reaction at such a low pH, it seems that the reaction reaches its equilibrium within a comparatively short period. After 15—20 minutes standing the excess of $CuCO_3$ suspension was centrifuged off and then each supernatant was measured by a Beckman type photo-electric spectrophotometer at 560 mμ. One per cent and 0.5 per cent egg albumin solutions were also examined in the same manner.

2. *Solubility Test*—0.5 per cent egg albumin solution (pH 7 by B.T.B.) was heated in a thermostat at 65°, 66° or 67° for several hours. Two ml. of the sample were pipetted out at an interval of 30 minutes, cooled down to the room temperature (15°) and then the same amount of $M/5$ acetate buffer (pH 4.8) was added to the sample each time. The precipitate was centrifuged off and 8 ml. of the strongly alkaline biuret test solution were added to 2 ml. of the supernatant. This solution was kept at 37° for 30 minutes and was measured spectrophotometrically at 530 mμ. (*4*).

The strongly alkaline biuret test solution was prepared as follows: Nine g. of potassium sodium tartrate were added to 400 ml. of $N/5$ NaOH solution in a 1000 ml. volumetric flask. Three g. of $CuSO_4 \cdot 5H_2O$ and 5 g. of KI were also added after the potassium sodium tartrate was completely dissolved, then $N/5$ NaOH solution was added again so as to bring the total volume of the mixed solution to 1000 ml.

The native sample was also treated in the same way. The value of the biuret reaction decreased as the amount of precipitate increased, namely, the denaturation proceeded. The degree of denaturation was obtained by subtracting the value of the biuret reaction of each supernatant from that of the native protein and plotted in terms of per cent of denatured component against time.

The data were shown in Fig. 3.

3. *Determination of the Reactivity of SH Groups*—The spectrophotometric method applied to this examination was based on the suggestion by Kajita. (*5*). A 0.6 per cent solution of egg albumin was mixed with the $M/2$ phosphate buffer solution (pH 6.8, checked by a glass electrode) in the proportion of 5 (protein solution): 1(buffer

solution)—3 (protein): 1 (buffer). This proportion was adjusted properly according to the concentration of the original solution of protein. Then $M/1000$ $K_3Fe(CN)_6$ (recrystallized and free of $Fe(CN)_6^{----}$ and Fe^{++}) was added to this mixed solution at the ratio of 1.0 ml. (protein-buffer solution): 0.2 ml. ($K_3Fe(CN)_6$). The sample thus prepared was heated at 67° or 70° in the same way with the above two cases, 1 ml. of which was pipetted out every 30 minutes, diluted by 5 ml. of the buffer solution so that the extinction be brought to the easiest measureable position.

This diluted sample was measured spectrophotometrically at 420 mμ where $K_3Fe(CN)_6$ gives the maximum absorption while the absorption by $K_4Fe(CN)_6$ is negligible. The extinction at 420 mμ decreased gradually as the reduction of $K_3Fe(CN)_6$ by the reactive SH groups proceeded. Each amount of SH groups oxidized was calculated so as to show per cent of cysteine, which was plotted against time and shown in Fig. 4.

RESULTS

1. Biuret Reaction

(*a*) *Horse Serum Albumin*—As is shown in Fig. 1, the denaturation followed the so-called stepwise course at 80°, while it proceeded along a smooth curve at 85°.

(*b*) *Egg Albumin*—A different type of curve was obtained for egg albumin at 75°, though a stepwise course was similarly observed at 65°. As far as we have tried, a smooth curve like horse serum albumin has not yet been obtained.

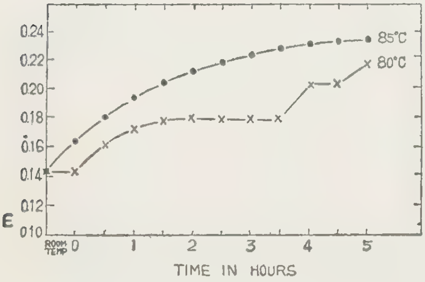

FIG. 1. Change in the reactivity of peptide bonds examined by biuret reaction. Sample: 1% horse serum albumin.

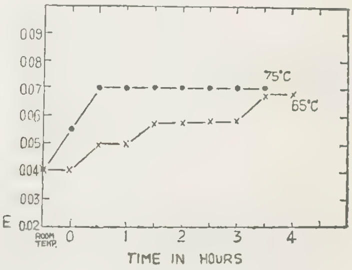

FIG. 2. Change in the reactivity of peptide bonds examined by biuret reaction. Sample: 0.5% egg albumin.

2. Solubility Test

Here as has been found to be the case with the above biuret reaction of horse serum albumin, 65° and 66° gave those stepwise curves and 67° showed a smooth curve. It is surprising that a difference of only one or two degree of temperature produces such a remarkable discrimination.

3. *Determination of the Reactivity of SH Groups*

We can observe the similar result again, *i.e.*, the stepwise nature was found at 67° and it turned smooth at 70° as is shown in Fig. 4.

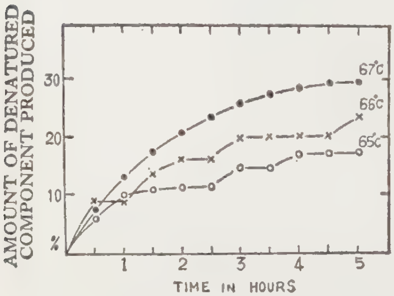

Fig. 3. Change in the amount of precipitation examined by solubility test. Sample: 0.5% egg albumin.

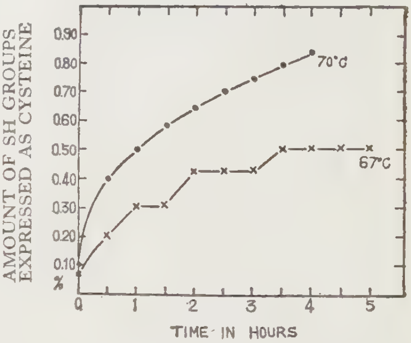

Fig. 4. Change in the reactivity of SH groups. Sample: 0.42% egg albumin.

When the sample was heated at 100° for 10 minutes with $K_3Fe(CN)_6$, SH groups which reacted with $K_3Fe(CN)_6$ amounted to 1.0–1.2 per cent though it varied slightly with the sample.

DISCUSSION

It may be said that the above investigations have demonstrated the stepwise nature of protein denaturation under appropriate conditions and agreed with the results observed by Tsyperovich (*6*), though our investigations were carried out from the different angles. However, several problems remain here:

(1) The biuret reaction and solubility test were carried out after the heated amples were cooled, which may give the denatured protein a chance to partially reverse itself, it is therefore necessary to take this into consideration whenever the interpratation is given to the above observations. The effect of cooling on the reversibility of heated protein will be detailed in the next report.

(2) As $K_3Fe(CN)_6$ solution reacted with protein while heated to-

gether, the effect of cooling could be omitted when we discuss the results obtained from the measurement of SH groups.

However, it might be assumed that $K_3Fe(CN)_6$ itself or the trace of impurities might disturb this heat denaturation process. Considering this possibility, the following examination was tried to make it sure:

The sample was separated into two flasks, $K_3Fe(CN)_6$ solution was added to one of them (I) and not to the other (II). These two were heated together under the same condition. One ml. of the sample of (I) was pipetted out every 30 minutes and measured in the usual manner, the extinction at 420 mμ decreased along a stepwise curve. Two hours later $K_3Fe(CN)_6$ was added to (II) at the same rate with (I), and both samples were measured at the same time within 10 minutes after $K_3Fe(CN)_6$ was added to (II). As is shown in Fig. 5, both values were exactly the same. This result has likely proved that $K_3Fe(CN)_6$ has not given any effect on the heat denaturation itself.

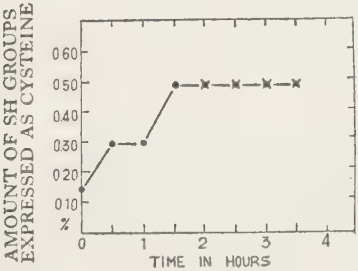

Fig. 5. Effect of $K_3Fe(CN)_6$ on heat denaturation.

Sample: 0.5% egg albumin.

•—• $K_3Fe(CN)_6$ reacted with protein from the beginning.

×—× $K_3Fe(CN)_6$ was added 2 hours later.

(3) As repeatedly mentioned above, it has clearly been demonstrated that the heat denaturation follows stepwise course under the proper conditions, of which the most important factor is a properly chosen temperature, *i.e.*, this stepwise nature has been shown at certain temperatures or below and turns smooth at the higher temperature. This fact likely gives as a deduction that the multifarious bonds in protein molecules are activated and brought to fission one after another by the milder activation, while if these bonds are activated by higher energies, the successive ruptures, probably because of their activation energies not so much different, proceed as if they were unimolecular.

Therefore, our final intention is to obtain the activation energy for each step, but unfortunately the protein denaturation, as far as we have tried and also as far as the above experiments are concerned, has little reproducibility, which has prevented us from attaining the aim.

As shown in Figs. 6, 7, and 8, even under the same condition, we have never obtained any reproducibility, even though the samples

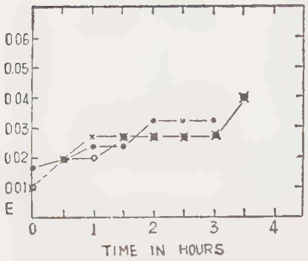

Fig. 6. Irreproducibility of heat denaturation examined by biuret reaction.
×—× 1st day (morning) ○—○ 1st day (afternoon) •—• 2nd day

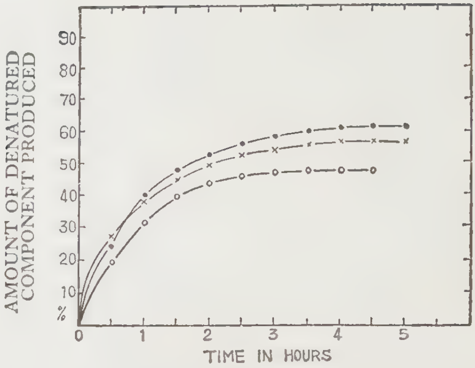

Fig. 7. Irreproducibility of heat denaturation examined by solubility test.
○—○ 1st day; ×—× 3rd day •—• 5th day

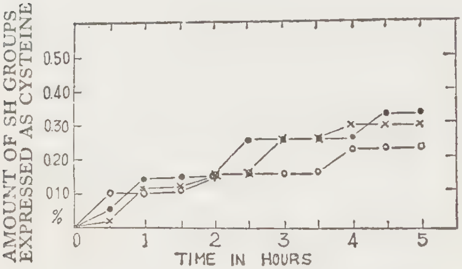

Fig. 8. Irreproducibility of heat denaturation examined by determination of reactivity of SH groups.
○—○ 1st day; ×—× 2nd day •—• 3rd day

have been kept in an ice-box and handled as carefully as possible all the time. The simultaneous examination of the same sample has, of course, given approximately the identical results. Those experiences gave us the conclusion that the protein molecule in the solution is more flexible than one can imagine, *i.e.*, the structure of protein in the solution is not so fixed but considerably changeable through aging.

The process of heat denaturation of protein was examined from the three different angles, *i.e.*, from the change in the reactivity of peptide bonds, from the change in the amount of precipitation at the iso-

SUMMARY

electric point, and from the change in the reactivity of SH groups.

In conclusion, the stepwise nature of the protein denaturation was revealed at the lower temperature, while the smooth curves were obtained at the higher temperatures.

Although the strict analysis or the exact deduction from those results is still beyond our reach, it is expected that this fact may have some suggestions and clue for the approach to the nature and mechanism of the protein denaturation.

The authors are indebted to the Ministry of Education for the Research Fund for Science.

REFERENCES

(*1*) Neurath, H., Greestein, J. P., Putnam, F. W., and Erickson, J. O., *Chem. Rev.*, **34**, 157 (1944)

(*2*) Araya, S., and Kobayashi, M., *J. Biochem.*, **38**, 7 (1951)

(*3*) Araya, S., and Iwasaki, M., *Seitai no Kagaku*, (in Japanese) **4**, 173 (1953)

(*4*) Saito, M., The laboratory technique for clinic by the method of photo-electric colorimetry (in Japanese), Nanzando, Tokyo 3rd Ed., p. 131 (1952)

(*5*) Kajita, A., *J. Jap. Biochem. Soc.*, **25**, 315 (1953)

(*6*) Tsyperovich, A. S., *Chem. Abstr.*, **46**, 8685 g (1952), cit. *Doklady Akad. Nauk S.S.S.R.* **83**, 257 (1952); *Chem. Abstr.*, **46**, 10221 b (1952), cit. *Ukrain Biokhim. Zhur.*, **20**, 108, 120 (1948)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

INHIBITORY EFFECT OF CYANINE AND RELATED DYES UPON CATALASE*

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(Received for publication, January 13, 1954)

During the last few years it has repeatedly been reported that certain cyanine and related dyes, which are known as photosensitizers, have fairly strong antimicrobial activities (*1*, *2*, *3*, *4*). The mechanism of this action, however, remains altogether obscure at present. It is, therefore, worth while, not only for the elucidation of the mechanism of this antimicrobial action but also from the purely enzymological viewpoint, to investigate the effects of these dyestuffs upon various enzymatic reactions. Among the many enzyme systems that have been studied, only cholinesterase were reported to be affected more or less strongly by some substances belonging to this group (*5*).

In the course of our studies dealing with the enzymatic oxidation of cyanine and related dyes by laccase, it was found that the action of catalase is strongly inhibited by some of these dyestuffs and moreover that there exists a distinct correlation between the oxidizability by laccase and the inhibitory effect upon catalase of these dyestuffs.

EXPERIMENTAL

The enzyme preparation and experimental methods used for determining the catalase reaction was virtually the same as described by Ogura and coworkers (*6*). In most cases the dyes were used as aqueous solutions. Only those dyes that are hardly soluble in water were used as solutions in one percent ethyl alcohol.***

All experiments were carried out at pH 7.0 and 0°C. Laccase was prepared

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[*] The first report of this study was made at the Symposium on Enzyme Chemistry held by the Chemical Society of Japan in Tokyo, in October, 1946.

[**] Present adress: Biological Institute, Faculty of Science, University of Nagoya.

[***] It has been shown that in this concentration ethyl alcohol has no effect upon catalase (*7*).

from *Lactarius piperatus* var. *pergameus* (=*Lactarius pergameus*)* and the oxidizability of dyes by the enzyme was determined by the disappearance of their original color, since oxidation of the dyes was always accompanied by the decolorization of the test solutions (*9*).

The results obtained are listed in the table, where the ϕ-values given in the column 3 indicate the concentration of dyes that caused 50 per cent inhibition of catalase reaction. As is evident from the table, some of cyanine dyes showed ϕ-values as low as $10^{-6.2} \sim 10^{-6.5}$ *mole/lit.*, which is of the same order of magnitude as the corresponding value obtained for potassium cyanide.

In column 4 in the same table are listed the susceptibility of these dyes to laccase. From these data it will be seen that only those dyes that are oxidized by laccase have inhibitory effect upon catalase.

DISCUSSION

Among a large number of substances ever known to inhibit catalase reaction, there are rather few that exert inhibitory action at the concentration as low as $10^{-6.0}$ *mole/lit.* From our results described above, some cyanine dyes should be included in this category. In their experiment already reported elsewhere, Ogura and coworkers (*10*) have found that neocyanine (No. 1 in the table) inhibits catalase in its dissociated form, namely as a cation; probably the same may be true for other members of cyanine dyes. This may be regarded as a characteristic feature of cyanine dyes as a catalase inhibitor, since all the poisonous substances—other than hydrogen ion—thus far known to inhibit catalase reaction had been shown to act either as anion or neutral molecule (*11*). Of interest is the fact that there is a distinct parallerism between the inhibitory effect on catalase and the oxidizability by laccase of the dyes. As regards the possible significance of our findings in relation to the antimicrobial effect of these dyes reference should be made to the report of Uemura (*4*), who studied the antibacterial action of cyanine dyes using *Staph. aureus* and *Escher. coli* as test organisms. One of the substances which were found to be strongly bacteriocidal was 1-methyl-1′-ethylbenzselena-2′-cyanine iodide (No. 12 in the table) which inhibited completely the growth of *Staph. aureus* at the concentration of $10^{-5.3}$ *mole/lit.* As may be seen from our table, however, this dye has no inhibitory action upon catalase at the same concentration. Pentamethine-cyanine (No. 10 in the table) was found to be more strongly bacteriocidal than neocyanines (No. 1 and No. 2 in the table), which

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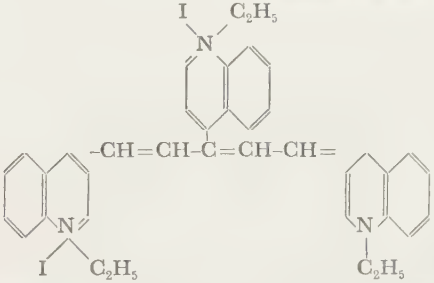
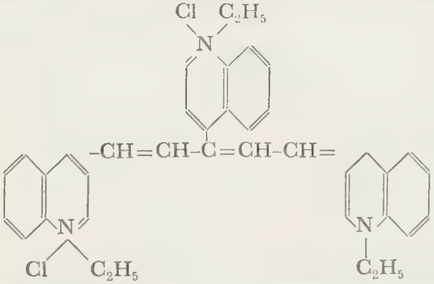
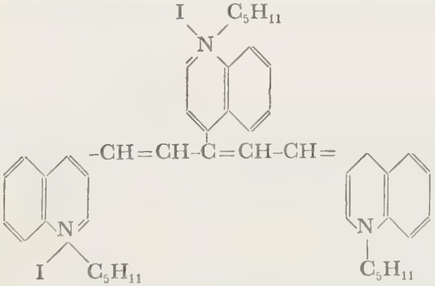
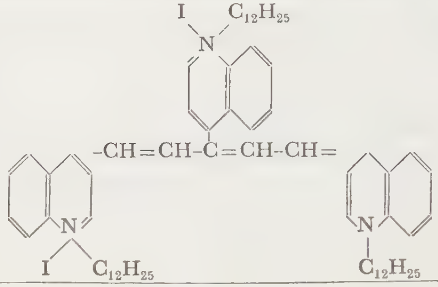
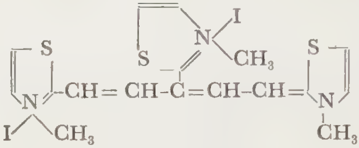
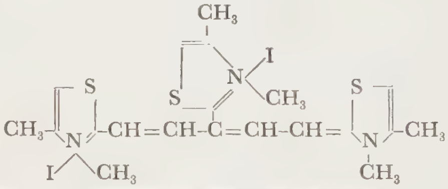
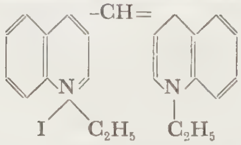
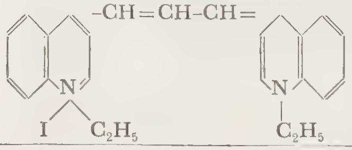
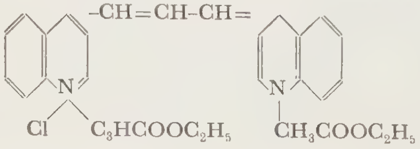
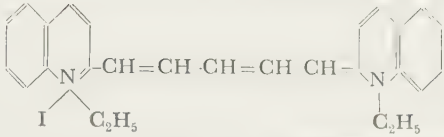
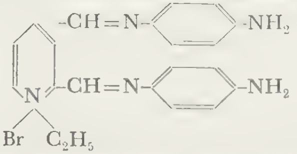
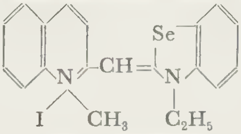
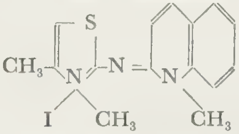
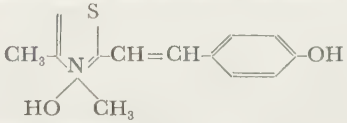
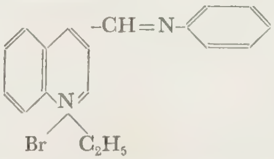
* This wild mushroom has hitherto been described as *Lactarius piperatutus* by Yakushiji (*8*) and us (*9*). Recently, however, Dr. K. Aoshima of the Governmental Experimental Station of Forestry, Tokyo, has kindly reinvestigated our mushroom and concluded that it is not authentic *L. piperatus* but its variety *pergameus* or *L. pergameus*.

TABLE I

Inhibitory Effect of Cyanine and Related Dyestuffs upon Catalase

No.	Chemical constitution	ϕ-Value (*mole/lit.*)	Oxidizability by laccase
1	 I C_2H_5 N -CH=CH-C=CH-CH= N I C_2H_5 N C_2H_5	$10^{-6.2}$	+
2	 Cl C_2H_5 N -CH=CH-C=CH-CH= N Cl C_2H_5 N C_2H_5	$10^{-6.3}$	+
3	 I C_5H_{11} N -CH=CH-C=CH-CH= N I C_5H_{11} N C_5H_{11}	$10^{-6.5}$	+

No.	Chemical constitution	ϕ-value (*mole/lit.*)	Oxidizability by laccase
4		$10^{-4.8*}$	+
5		$10^{-3.5}$	+
6		$10^{-3.6}$	+
7		$10^{-3.5}$	+
8		$10^{-5.4}$	+
9		$10^{-4.2}$	+

No.	Chemical constitution	ϕ-value (*mole/lit.*)	Oxidizability by laccase
10		*$10^{-3.9}$*	+
11		$10^{-3.6}$	+
12		—**	−
13		—	—
14		—	—
15		—	—

* Italic from of the values shows that the experiments were performed with one percent alcohol solution. For the effect of alcohol on catalase, see (*7*).

** No inhibition could be observed even at the concentration of $10^{-3.0}$ *mole/lit.*

have, however, stronger inhibitory action upon catalase reaction than the former. It seems, therefore, likely that the effect of the dyes upon catalase has little to do with their antibacterial action.

SUMMARY

Using fifteen kinds of cyanine and ralated dyes, their effect upon the action of catalase was investigated. Some compounds were found to be strongly inhibitory, while others displayed no such action. The strongest inhibitors showed their effect in a concentration as low as $10^{-6.5}$ *mole/lit.* It was found that there is a distinct parallelism between the inhibitory effect upon catalase and the oxidizability by laccase of the dyes.

The authors wish to express their sincere gratitude to Prof. H. Tamiya and Mr. G. Shibata for their encouragement and advices throughout the course of this study. The dyestuffs used were kindely supplied by Prof. E. Ochiai of the Department of Pharmacy, University of Tokyo, and Dr. T. Ogata of the Scientific Research Institute, Ltd., to whom we wish to acknowledge our indebtedness. Thanks are also due to the Ministry of Education for a grant which enabled this reaserch to be carried out.

REFERENCES

(*1*) Brooker, L.G.S., and Sweet, L. A., *Science*, **105**, 496 (1947)

(*2*) Wright, H. N., Litchfield, J. T., Jr., Brey, T., Cranston, E.M., Cuckler, A. C., and Bieter, R. N., *Ann. N. Y. Acad. Sci.*, **50**, 109 (1948)

(*3*) Bueding, E., *Ann. N. Y. Acad. Sci.*, **50**, 115 (1948)

(*4*) Uemura, T., *J. Antibiotics* (*Japan*), **2**, 512 (1949); *Bot. Mag.* (*Tokyo*), **66**, 63 (1953)

(*5*) Hase, E., Mizuno, Y., and Katayanagi, M., *Bull. Chem. Soc. Japan*, **22**, 250 (1949)

(*6*) Ogura, Y., Tonomura, Y., Hino, S., and Tamiya, H., *J. Biochem.*, **37**, 153 (1950)

(*7*) Tonomura, Y., and Ogura, Y., *Bot. Mag.* (*Tokyo*), **63**, 288 (1950)

(*8*) Yakushiji, E., *Acta Phytochim.*, **12**, 227 (1941)

(*9*) Hino, S., Fujita, M., and Ogura, Y., *J. Biochem.*, **39**, 101 (1952)

(*10*) Ogura, Y., Hattori, A., Tonomura, Y., and Hino, S., *Symp. Enz. Chem.* (in Japanese), **5**, 28 (1950)

(*11*) Ogura, Y., Tonomura, Y., and Hino, S., *Chem. Researches*, (in Japanese), **4**, 73, (1948)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

BILE ACIDS AND STEROIDS

III. BROMINATION OF 7-KETOCHOLANIC ACID DERIVATIVE

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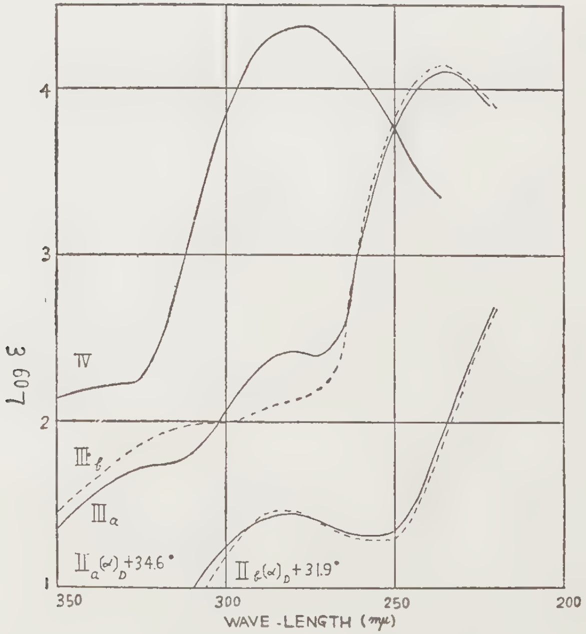
(Received for publication, January 21, 1954)

In the bromination of 7-keto-steroids, it has been known that bromine atom entered the position of C_6. For instance, Barr, *et al.* (*1*) reported that α and β epimers of 6-bromo-compound were obtained by bromination of 7-keto-cholestanyl acetate. Dane and Wulle (*2*) found that a bromo-compound, which was not affected by the elimination reaction of the hydrogen bromide by pyridine but was converted into 7-keto Δ^5-cholenic acid by pyridine and silver nitrate, was prepared from 7-ketocholanic acid. Lardon (*3*) reported that methyl 3,12-diacetoxy-7-ketoetiocholanate gave also 6-bromo-compound, which gave 7-keto $\Delta^{3,5}$-etiocholanic acid by pyridine and silver nitrate. But on the bromo-compounds of ethyl 3,12-diacetoxy-7-ketocholanate, there was only a simple reference of Hoehn and Linsk (*4*). They reported that the bromo-compound of m.p. 130° was isolated but hydrogen-bromide could not be eliminated by pyridine.

Therefore, the bromination of ethyl 3,12-diacetoxy 7-ketocholanate (I) in glacial acetic acid was attempted and gave some interesting results. These are described in the present paper.

The bromination of ethyl 3,12-diacetoxy-7-ketocholanate (I) in glacial acetic acid at warm temperature by drop-wise addition of bromine gave prismatic bromo-compound (IIa) of m.p. 134–136°, 34.5 per cent yield and needle-like bromo-compound (IIb) of m.p. 160–162°, 18 per cent yield. When bromine was added at one time to the reaction mixture at low temperature, the yield of (IIa) increased to 72 per cent but the yield of (IIb) decreased to about 5 per cent, on the contrary. These bromides showed very similar optical rotations and ultraviolet spectra (Fig. 1), and so they might not be assumed to be the epimer of C_6. Infrared spectra, showed that vibrations of 7-keto-groups remove from 5.83 μ to 5.76 μ which indicated that the both bromo compound belong to α-bromo compound (*5*). The great difference was found, however, in their elimination products of hydrogen-bromides. Refluxing with pyridine could not eliminate hydrogen-bromide. The elimination was accomplished only by pyridine and silver-nitrate. (IIa) gave the prism of m.p. 166–168° (IIIa), and (IIb) gave the needles of m.p. 211–213° (IIIb) respectively. Both of their ultraviolet spectra have the strong absorption at 235 mμ and 236 mμ, respectively ($\log\varepsilon$ 4.10, 4.14) and represent the characteristic type of α,β-unsaturated keton, but there is a difference that (IIIa) has an only slight shoulder at 279 mμ (Fig. 1). In both infrared spectra, the vibrations of 7-keto-group remove to 5.98 μ. From the presence of the conjugated double bond at 6.15 μ it is assumed that both (IIIa) and (IIIb) should be α,β-unsaturated keto-derivatives.

The saponification of (IIIa) by potassium hydroxide and alcohol gave the needle-like crystal of m.p. 202–205° (IV), which has a maximum absorption at 278 mμ ($\log\varepsilon$ 4.38) (Fig. 1) characteristic of dienone in the absorption spectrum. As the result, it is assumed that 3α-acetoxy group would have been eliminated as acetic acid and (IV) should be 12-hydroxy-7-keto-$\Delta^{3,5}$-choladienic acid which is identical with the

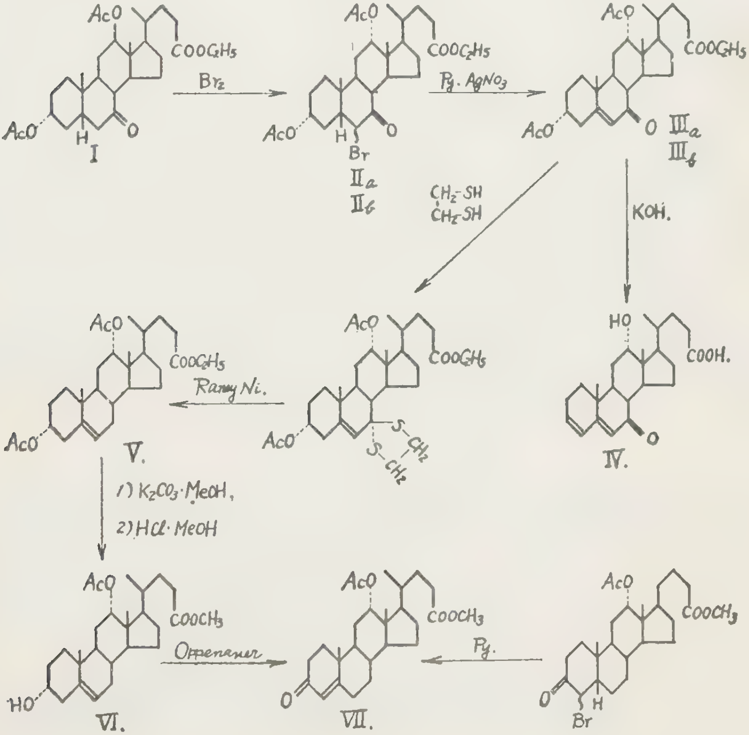


Ultraviolet absorption spectra in 94% ethylalcohol.

compound which Baba and Kazuno prepared (*6*). The saponification of (IIIb) also gave the same needle-like crystal, and a mixed melting point with (IV) showed no depression.

In view of the above results, if it were assumed that both bromides, (IIa) and (IIb), would be epimers of C_6, their elimination products, (IIIa), and (IIIb), would accord with one another. If it were presumed that either (IIa) or (IIb) would be 8-bromocompound, its elimination product would be 7-keto-$\Delta^{8(9)}$ (or $\Delta^{8(14)}$)-compound, which must show a maximum at about 250 mμ in the absorption spectra.

However, it was found by L. F. Fieser (*7*) that when methyl 3-carbethoxy- (or acetoxy-) 7,12-dihydroxy cholanate was converted into 7-keto-derivatives by oxidation, it gave by several oxidation reagents various isomeric derivatives melting points and which showed different specific rotations but resembled very much in infrared spectra each



other. As our result was similar to that phenomenon, the necessity of comparing both substances with each other after reduction of ketones was reconegnized.

To reduce the keto-groups of α,β-unsaturated ketones, the violent condition which caused the inversion like Wolff-Kishner reduction (*8*) was avoided and the reduction by Raney-Nickel after condensation with ethandithiol, which H. Hauptmann (*9*) described, was used. Consequently both substances gave the prismatic crystal of m.p. 170–172° (V), and a mixed melting point with one another showed no depression, and both reduction products showed the lapse of keto-groups and no absorption in ultraviolet spectra. In the reduction process, the crude material was used as the condensation products with ethaneditiol was difficult to purify.

To confirm the structure of (V), we obtained the needle-like crystals of m.p. 130–132° (VII) by esterification and Oppenauer oxidation after partial saponification which gave no color reaction with tetranitromethane ((V), and (VI) gave positive reaction with this reagent). The mixed melting point with methyl 3-keto-12-acetoxy-Δ^4-cholenate prepared from 3-keto-4 bromo-compound by the method of T. Reichstein (*10*), showed no depression, and the ultraviolet spectrum has the absorption maximum at 240.5 mμ (logε 4.21), characteristic of α,β-unsaturated ketone.

As the results of the above-stated, it was considered that two kinds of bromides and dehalogenated products were 7-keto-6-bromo-compounds and 7-keto-Δ^5-unsaturated compounds respectively, but could not be concluded what isomers they were.

The authors wish to thank Messrs. Ieki and Miyahara for taking charge of elemental analyses and to Messrs. Kubota, Inaba, and Matsui for taking change of spectral measurements.

EXPERIMENTAL

All melting points are uncorrected.

(*1*) *Ethyl 3,12-diacetoxy-7-keto-6-bromocholanate* (*IIa*) *and* (*IIb*)—(A) 20 mg. of ethyl 7-keto-3,12-dihydroxycholanate dissolved in 40 ml. of acetic anhydride and 40 ml. of pyridine was warmed on a steam-bath for 2 hours, and the excess of acetic anhydride and pyridine was evaporated *in vacuo* as possible. Methanol was added to decompose acetic anhydride and evaporated to dryness *in vacuo*. The residue dissolved in 200 ml. of glacial acetic acid was warmed at 55–60°, and 7.4 g. of bromine dissolved in 50 ml. of glacial acetic acid was then added in drop-wise for one hour. Then, reaction mixture was mechanically stirred for 30 minutes, poured into ice-water, and extracted with ether. The ethereal solution was washed with water, 5 per cent sodium bicarbonate solution, and again water, dried over with sodium sulfate and evaporated. The residue was twice recrystallized from methanol, and there was obtained by filtration 9.5 g. of prismatic crystal, m.p. 134–136° (IIa), yield, 34.5 per cent, $\lambda^{\text{alc.}}_{\text{max.}}$ 282 mμ (logε 1.46).

Analysis Calcd. for $C_{30}H_{45}O_7Br$: C 60.29, H 7.59
Found C 60.43, H 7.55

After the mother liquor was evaporated to dryness, it was warmed on a steam-bath with acetic anhydride and pyridine for 4 hours, and 13 g. of the neutral residue was obtained by extraction and chromatographed from the solution of benzenepetroleum-ether (1 : 1) over alumina. From the eluate of benzene-petroleum ether (1 : 1), 5 g. of needles, m.p. 160–162° (IIb) was yielded. Recrystallization from methanol, or ether-petroleum-ether, $\lambda^{\text{alc.}}_{\text{max.}}$ 282 mμ (logε 1.46).

Analysis Calcd. for $C_{30}H_{45}O_7Br$: C 60.29, H 7.59
Found C 60.59, H 7.56

(B) 7 g. of ethyl 3,12-dihydroxy 7-ketocholanate was treated in the same way as above with 15 ml. acetic anhydride and 15 ml. of pyridine, and then 2.6 g. of bromine in glacial acetic acid was added. The mixture was allowed to stand overnight under cooling with water, and then 7.0g. of (IIa) was collected from methanol by recrystallization. The mother liquor was treated as mentioned above, and the crystals of m.p. 145–147° were twice recrystallized from methanol and there was obtained about 500 mg. of (IIb), m.p. 160–162°.

(2) *Ethyl 3,12-diacetoxy-7-keto-Δ^5-cholanate* (*IIIa*), *and* (*IIIb*)—(A) 6 g. of (IIa) and 18 g. of silver-nitrate were dissolved in pyridine. The reaction mixture was refluxed at 130° for 11 hours, and then pyridine was evaporated under reduced pressure. The residue was poured into water and extracted with ether. Ethereal solution was washed with 5 per cent nitric acid, 5 per cent sodium bicarbonate solution, and then water, dried over with sodium sulfate, concentrated, and allowed to stand. 2.2 g. of crystal which was precitated was twice recrystallized from methanol to 1.9 g. of prismatic crystal, m.p. 166–168° (IIIa). From the mother liquor of ethereal solution, a little of the material was recovered, $\lambda^{\text{alc.}}_{\text{max.}}$ 235 mμ (log ε 4.10), 279 mμ (log ε 2.42).

Analysis Calcd. for $C_{30}H_{44}O_7$: C 69.74, H 8.58
Found C 69.67, H 8.50

(B) The above treatment with 1 g. of (IIb) gave the crystal of m.p. 200–209°. Recrystallization from methanol or alcohol gave 0.3 g. of needles, m.p. 211–213° (IIIb), $\lambda^{\text{alc.}}_{\text{max.}}$ 236 mμ (log ε 4.14).

Analysis Calcd. for $C_{30}H_{44}O_7$: C 69.74, H 8.58
Found C 69.47, H 8.46

(3) *7-Keto 12-hydroxy-$\Delta^{3,5}$-choladienic Acid* (*IV*)—(A) 100 mg. of (IIIa) and 100 mg. of potassium hydroxyde dissolved in 10 ml. of diluted alcohol were saponified on the steam-bath for 2 hours. Recrystallization from diluted alcohol gave the needle-

like crystals of m.p. 202–205°, $\lambda_{\text{max.}}^{\text{alc.}}$ 278 mμ (log ε 4.38).

Analysis Calcd. for $C_{24}H_{34}O_4$: C 74.58, H 8.87
Found C 74.39, H 9.06

(B) Saponification of (IIIb) gave the needles of m.p. 202–205°, and a mixed melting point with the sample prepared from (IIIa) showed no depression.

Analysis Calcd. for $C_{24}H_{34}O_4$: C 74.58, H 8.87
Found C 74.43, H 9.31

(4) *Ethyl 3,12-diacetoxy-Δ^5-cholenate* (*V*)—(A) 500 mg. of (IIIa), 500 mg. of ethandithiol, 5 g. of fused zinc chloride, and 5 g. of dehydrated sodium sulfate were added to 12ml. of dioxane, allowed to stand overnight, and extracted with ether. The ethereal solution was washed with water, diluted hydrochloride, diluted sodium bicarbonate solution, and again water, dried over with sodium sulfate, and evaporated. The residue dissolved in diluted alcohol was allowed to stand. Recrystallization from diluted alcohol or diluted methanol gave 400 mg. of the needle-like crystals of m.p. 84–86°. This was positive in sulphur test and colored yellow with tetranitromethane.

Analysis Calcd. for $C_{32}H_{48}O_6S_2$: C 64.83, H 8.16
Found C 64.61, H 8.27

This substance and 10 g. of Raney nickel prepared freshly were warmed in 10 ml. of dioxane on the steam-bath for 14 hours. Then the nickel was filtered off and the solvent was evaporated *in vacuo*. Twice recrystallization form methanol gave 200 mg. of the prism, m.p. 170–172°. This was positive in tetranitromethane test and free from S.

Analysis Calcd. for $C_{30}H_{46}O_6$: C 71.68, H 9.22
Found C 71.93, H 8.82

(B) 1 g. of (IIIb), 1.5 g. of ethandithiol, 10 g. of dehydrated sodium sulfate, and 10 g. of fused zinc chloride were added to 30 ml. of dioxane, allowed to stand for 5 days, extracted with benzene, and chromatographed on alumina in benzene solution. The eluate of benzene gave 500 mg. of the crystal of m.p. *ca.* 80° from dilute methanol. This substance and 10 g. of freshly prepared Raney nickel were warmed with 30 ml. of dioxane on the steam-bath, and treated as mentioned above and the prism of m.p. 169–171°. It gave a positive tetranitromethane test but sulphur test was negative and a mixed melting point with the sample from (IIIa) showed no depression.

Analysis Calcd. for $C_{30}H_{46}O_6$: C 71.68, H 9.22
Found C 71.38, H 9.23

(5) *Methyl 3-Hydroxy-12-acetoxy-Δ^5-cholenate* (*VI*)—200 mg. of (V) and 110 mg. of potassium bicarbonate dissolved in 30 ml. of diluted methanol were warmed on the steam-bath for 2 hours. The reaction mixture was then diluted with water and acidified with hydrochloric acid. The free acid precipitated was collected by filtration, and esterified with 1 per cent of hydrochloride methanol. The recrystallization from methanol gave 100 mg. of methyl 3-hydroxy-12-acetoxy-Δ^5-cholenate (VI), m.p.

207–210°. This was positive for tetranitromethane.

Analysis Calcd. for $C_{27}H_{42}O_5$: C 72.61, H 9.48
Found C 72.31, H 9.48

(6) *Methyl 3-keto-12-accetoxy-Δ^4-cholenate* (*VII*)—70 mg. of (VI) and 200 mg. of aluminium-isopropoxide dissolved in 20 ml. of benzene and 2 g. of acetone. The reaction mixture was refluxed for 25 hours, then poured into diluted hydrochloric acid, and extracted with benzene. The benzene solution was washed with water, diluted sodium bicarbonate solution, and again water, dried over with sodium sulfate and evaporated. The residue was not crystallized, so chromatographed on alumina in benzene solution. The benzene eluate gave about 10 mg. of the needle-like crystals of m.p. 130–132°. This was negative for tetranitromethane, $\lambda_{\text{max.}}^{\text{alc.}}$ 240.5 mμ (log ε 4.21). A mixed melting point with the authentic sample prepared from desoxycholic acid *via* methyl 3-keto-4-bromo-12-acetoxycholanate by method of T. Reichstein (*9*) showed no depression.

Analysis Calcd. fo. $C_{27}H_{40}O_5$: C 72.94, H 9.07
Found C 72.71, H 8.84

SUMMARY

Bromination of ethyl 3,12-diacetoxy-7-keto-cholanate in glacial acetic acid gave two kinds of bromides. These gave also different dehalogenation products which yielded the same substance by saponification. When these two isomeric dehalogenation products were reduced to non-ketonic substances, these gave only one product. By conversion of the reduction product to methyl 3-keto-12-acetoxy-Δ^4-cholenate, it was assumed that both dehalogenation products were 7-keto-Δ^5-compounds and the bromine atom was substituted at C_6 in both bromides.

REFERENCES

(*1*) Barr, T., Heilbon, I. M., Jones, E. R. H., and Spring, F. S., *J. Chem. Soc.*, 334 (1938)
(*2*) Dane, E., and Wulle, H., *Z. physiol. Chem.*, **267**, 1 (1941)
(*3*) Lardon, A., *Helv. Chem. Acta*, **30**, 597 (1947)
(*4*) Hoehn, W. M., and Linsk, J., *J. Am. Chem. Soc.*, **67**, 312 (1945)
(*5*) Jones, R. N., Ramsay, D. A., Herling, F., and Dobriner, K., *J. Am. Chem. Soc.*, **74**, 2828 (1952)
(*6*) Baba, T., and Kazuno, T., *J. Biochem.*, **39**, 7 (1952)
(*7*) Fieser, L. F., and Rajago palan, S., *J. Am. Chem. Soc.*, **72**, 5530

(1950)

(*8*) Huang Minlon, *J. Am. Chem. Soc.*, **68**, 2487 (1946)

(*9*) Hauptmann, H., *J. Am. Chem. Soc.*, **68**, 562 (1947)

(*10*) Barck Hardt, V., and Reichstein, T., *Helv. Chim. Acta*, **25**, 821 (1942); Wendler, K. L., and Reichstein, T., *Helv. Chim. Acta*, **31**, 1713 (1948)

The Journal of Biochemistry. Vol. 41, No. 3. 1954

ZUR KENNTNIS DER METHYLIERUNG DES PYRIDINS IM KANINCHENORGANISMUS

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(Der Schriftleitung zugegangen am 22. Januar 1954)

Dass dem tierischen Organismus die Fähigkeit der Methylierung zukommt, ist bekannt. Das Vorkommen methylierter Stoffwechselprodukte wie des Kreatins und das Auftreten von Methylpurinen im Urin normalerweise sprachen ohne weiteres in diesem Sinne. Viele Hormone, Vitamine und Pigmente sind Verbindungen, die an N- oder C-Atomen Methylgruppen enthalten. Es sind auch Beispiele bekannt, wo der Eintritt von Methylgruppen als eine Entgiftung toxischwirkender Substanzen aufgefasst werden muss.

Seit der Feststellung von W. His (*1*), wonach verabfolgtes Pyridin als Methylpyridylammonium-hydroxyd zur Ausscheidung gelangt, ist das Verhalten des Pyridins bei verschiedenen tierischen Organismen von mehreren Autoren (*2*) studiert worden. Es ist dabei sehr merkwürdig, dass nur das Kaninchen, in dessen Harn das Ammoniak in sehr geringer Menge ausgeschieden wird, eine ganz schwache Fähigkeit hat, das aufgenommene Pyridin in die Methylverbindung überzuführen.

Über die Bedingungen, unter denen es zur Bildung methylierter Verbindungen kommt, sind wir aber noch sehr wenig unterrichtet. In Berücksichtigung der besonderen Beziehung zwischen Ammoniakbildung und dem Methylierungsvermögen des Pyridins im Kaninchenorganimus scheint es uns wahrscheinlich, dass die Intensität der Amoniakbildung jedenfalls bei der Methylierung der eingeführten Verbindungen eine wesentliche Rolle spielt.

Zieht man nun die Frage in Betracht, aus welchen Substanzen das Methylradical im tierischen Körper gebildet wird, so ergibt sich vor allem eine grosse Wahrscheinlichkeit für Glykolsäure, Glyoxylsäure und Methionin. Die Desaminierung des Glykokolls muss entweder hydrolytisch oder oxydativ verlaufen. Die erwartungsgemäss als Zwischenprodukte auftretenden Verbindungen (Glykolsäure und Glyoxylsäure) liefern durch Entcarboxylierung den Methylalkohol und Formaldehyd.

Wenn es sich nun zeigt, dass sich im Tierkörper durch Desaminierung des Glykokolls die Ammoniakbidung einerseits und Methylradicalfreiheit anderseits gleichzeitig vollziehen, so wird die Methylierung des verabreichten Pyridins im Kaninchen-organismus sehr schwer zugänglich sein, weil das Ammoniak im Urin dieses Tieres nur in sehr geringer Menge ausgeschieden wird.

In diesem Zusammenhange schien es uns einer exakten experimentellen Prüfung würdig zu sein und ich habe auf Veranlassung von Herrn Prof. M. Tomita die Fütterungsversuche des Pyridins mit und ohne Glykolsäurezusatz an Kaninchen angestellt, um die Rolle der letzteren Verbindung auf die Methylierung des Pyridins aufzuklären. Es ergab sich dabei, dass sich die Ausscheidung des Methylpyridylammoniumhydroxyds im Urin des Versuchstieres bei der gleichzeitigen Zugabe von Glykolsäure auffallenderweise vermehrt.

Bei dieser Untersuchung habe ich meine Aufmerksamkeit zugleich nach zwei Richtungen geschenkt, einerseits um zu klaren, wie der Methylierungsvorgang von der Aussentemperatur abhängig ist, und andererseits die Kenntnis der Adaptationserscheinung des Kaninchens gegen Vergiftung des Pyridins zu erweitern. Bei der monatelangen dauernden Verabreichung des Pyridins ist es festgestellt, dass das Methylierungsvermögen dieser Verbindung im Kaninchenorganismus allmählich gesteigert wird.

EXPERIMENTELLE BELEGE

1. *Versuchsreihe*

Versuch 1: Kaninchen wurde mit Grass gefüttert und mit 0.25 g. Pyridin täglich oder 1 mal alle 2 Tage als essigsaures Salz in 5 proz. Lösung subcutan injiziert. In 65 Tagen (30. IX. 1951–3. XII. 1951) wurde 9 g. Pyridin verfüttert. Der Harn wurde mit neutralem essigsaurem Blei im Überschuss gefällt, und nach Absetzen des Niederschlages von diesem abgegossen, durch Zusatz von basischessigsaurem Blei und Ammoniak von neuem ausgefällt und filtriert. Das Filtrat wurde durch Schwefelsäure vom Blei befreit, filtriert, dann unter vermindertem Druck eingeengt und mit Kaliumquecksiberjodid-lösung versetzt. Nach 24 Stunden wurde der Niederschlag anf dem Filter gesammelt und sorgfältig ausgewaschen. Der Niderschlag wurde unter Zusatz von Schwefelsäure durch Silberoxyd zerlegt. Nach Abfiltrieren vom Jodsilber wurde das Filtrat durch Barytwasser von überschüssiger Schwefelsäure und Silbersulfat befreit, der überschüssige Baryt durch Einleiten von Kohlensäure und Erwärmen entfernt und die wässerige Lösung mit Salzsäure genau neutralisiert. Jetzt wurde die Lösung unter vermindertem Druck eingedampft and mit Alkohol ausgezogen. Aus der alkoholischen Lösung wurde mittels Platinchlorides das Doppelsalz der Base isoliert. Das Platinchloriddoppelsalz wurde mit Alkohol und Aether gewaschen und aus heissem Wasser umkristallisiert. Es betrug 0.0542 g.

Versuch 2: Seit dem 30. IX. 1951 wurde einem Kaninchen täglich oder 1 mal alle zwei Tage 0.25 g. Pyridin als essigsaures Salz in 5 proz. Lösung subcutan injiziert. Die Fütterung wurde in derselben Weise bis 3. XII. 1951 fortgesetzt. Der Harn wurde auf gleiche Weise wie beim *Versuch 1* behandelt. Im ganzen wurden 9 g. Pyridin verfüttert. Das Platinchloriddoppelsalz betrag 0.0242 g.

Mit demselben Tier wurde weiterer Fütterungsversuch fortgesetzt. Seit 4. XII. 1951 wurde dem Kaninchen wieder 1 mal alle zwei Tage 0.25 g. Pyridin als essigsaures Salz in 5 proz. Lösung subcutan injiziert. In 99 Tagen wurde das Kaninchen 9.45 g. Pyridin verfüttert. Der Harn wurde in bekannter Weise auf Methylpyridylammoniumhydroxyd verarbeitet. Es wurde erhalten : 0.1180 g. Platinchloriddoppelsalz.

Die Ergebnisse gehen aus folgender Übersicht hervor.

TABELLE I

Kaninchen	Datum	Zugeführtes pyridin	Platinchloriddoppelsalz des methylpyridylammoniumhydroxyds
Nr.		*g.*	*g.*
1	30. IX-3. XII (1951)	9.00	0.0542
2	30. IX-3. XII (1951)	9.00	0.0242
//	4. XII-3. III (1951-1952)	9.45	0.1180

2. *Versuchsreihe*

Versuch 3: 3 Kaninchen, die normalerweise gefüttert waren, wurden je 0.25 g. Pyridin und 0.25 g. Glykolsäure in 5 proz. wässeriger Lösung 1 mal alle 2 oder 3 Tage subcutan injiziert. Der Harn wurde auf gleiche Weise beim *Versuch 1* behandelt. Im ganzen wurden 7 g. Pyridin und 7 g. Glykolsäure in 22 Tagen an 3 Kaninchen

TABELLE II

Kaninchen	Datum	Zugeführte Subst.		Platinchloriddoppelsalz des Methylpyridylammoniumhydroxyds
		Pyridin	Glykolsäure	
Nr.		*g.*	*g.*	*g.*
3	11.V-2.VI (1952)	2.125	2.125	0.1865
4	11.V-2.VI (1952)	2.500	2.500	
5	11.V-2.VI (1952)	2.375 7.000 (+	2.375 7.000 (+	

verfüttert. Man erhielt 0.1865 g. Platinchloriddoppelsalz, dasi n orangeroten Tafeln kristallisierte und bei 207° schmolz.

Tabelle II veranschaulicht die Resultate.

Wie aus Tabelle I und II zu ersehen ist, wird das Methylierungsvermögen des Pyridins in Kaninchen-organismus bei der monatelangen dauernden Verabreichung etwas gesteigert (*Versuch 2.*) und die Ausscheidung des Methylpyridylammoniumhydroxyds in Kaninchenharn vermehrt sich auffallenderweise bei der gleichzeitigen Zugabe von Glykolsäure (*Versuch 3, 4, 5,*).

LITERATUR

(*1*) His, W., *Arch. exper. Pathol. u. Pharm.*, **22**, 253 (1887)

(*2*) Hoshiai, A., *Z. physiol. Chem.*, **62**, 118 (1909); Hoshisi, Z., und Totani, G., *Z. physiol. Chem.*, **68**. 83 (1910); Mayeda, K, und Ogata, M., *Z. physiol. Chem.*, **89**, 251 (1914); Abderhalden, E., Brahm und Schttenhelm., *Z. physiol. Chem.*, **59**, 32; (1909); **62**, 133 (1909); Tomita, M., *Biochm. Z.*, **116**, 48 (1921); **116**, 55 (1921)

The Journal of Biochemistry, Vol. 41, No. 3, 1954

BEITRÄGE ZUR KENNTNIS DER ORNITHURSÄURE-BILDUNG IM VOGELORGANISMUS

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(Der Schriftleitung zugegangen am 22. Januar 1954)

Während bei der Fütterung von Säugetieren mit Benzoesäure oder Phenylessigsäure bekanntlich Hippursäure oder Phenacetursäure im Harn ausgeschieden werden, verwandeln jene Säure sich dagegen bei Hühnern nach der Entdeckung von M. Jaffé (1877) und G. Totani (1910) in Ornithursäure oder Phenylacetylornithin.

Da die Analogie, welche die Substanzen der Pyridingruppe in ihrem chemishen Charakter mit den Benzolderivaten darbieten, zu der Erwartung berechtigte, dass sie auch im Tierkörper ein ähnliches Verhalten zeigen würden, wie die aromatischen Verbindungen, so hat Sendju (1927) das Schicksal der α-Picolinsäure im Vogelorganismus verfolgt und ergeben, dass sich diese Säure bei Hühnern in Dipicolylornithin verwandelt. Anderseits hat M. Takahashi (1928) unter Leitung von M. Tomita geprüft, inwieweit schon beim Fötus die Zeichen der Ornithinpaarung von hinzugeführter Benzoesäure nachweisbar sind. Es zeigte sich dabei, dass die Ornithursäure als ein Entgiftungs-produckt (im Hühnerorganismus) schon nach 14 tägiger Bebrütung gebildet und in Allantois-flässigkeit ausgeschieden wird.

Da das Ornithin in erster Linie eine unentbehrliche Verbindung bei dem Stickstoffumsatz ist, so ist wohl anzunehmen, dass die Ornithinpaarung der verabreichten Giftstoffe dem ganzen Stoffwechsel abhängig sei. In der Tat standen sich die Angaben in bezug auf die Stärke der Ornithin-paarung bei der Fütterung der oben beschriebenen Verbindungen gegenüber. In unserem Laboratorium begegneten wir sogar oft zu den negativen Ergebnissen.

Um die engeren Beziehungen zwischen der Ornithursäure-bildung und dem allgemeinen Stoffwechsel kennenzulernen, haben wir das ganze Jahr hindurch eine Reihe von Untersuchungen über Ornithin-paarung bei der Fütterung von Benzoesäure im Hühnerorganismus angestellt, deren Resultate im folgenden mitgeteilt werden.

EXPERIMENTELLE BELEGE

Isolierung der Ornithursäure

Die Versuche wurden seit August 1951 bis Juli 1952 ausgeführt. Bei jeden Versuchen brauchte man 5–6 Hühner. Einem Tier wurde Benzoesäure als Natriumsalz in wässeriger Lösung in täglich einmaliger Dosis von 0.5–1.0 g verabreicht. Die nach Eingabe von Na-benzoat gesammelten Exkremente (der Hühner) wurden auf dem Wasserbad getrocknet und mit heissem Alkohol extrahiert. Der Alkoholextrakt wurde unter vermindertem Druck verdunstet, der Rückstand mit 5 proz. H_2SO_4 aufgenommen und mit Essigester ausgezogen. Der Essigätherauszug liess nach dem Eindampfen unter vermindertem Druck eine wenig gefärbte Kristallmasse zurück. Diese wurde dann mit Petroläther extrahiert. Die in Petroläther unlösliche Substanz wurde in heissem Alkohol aufgenommen und im Eisschrank stehen gelassen. Daraus schied sich allmählich gut kristallisierte Ornithursäure ab. Ihr Schmelzpunkt liegt bei 184°:

Die Ergebnisse sind in der Tabelle kurz verzeichnet.

TABELLE I

Versuchsmonate	Verabreichte Menge von Na-benzoat (0.5–1.0 g. pro die, per Kopf)	Ausbeute der erhalten Ornithursäure	
August 1951 September 1951	10.0 *g*	0.03 *g*.	0.30%
Oktober 1951	4.0	0.16	4.00
November 1951	8.0	0.31	3.87
Dezember 1951 Januar 1952	4.0	0.18	4.50
Februar	4.0	0.26	6.50
März 1952	4.0	0.91	22.75
April 1952	4.0	0.13	3.25
Mai 1952	4.0	(0.00)	(0)
Juni 1952 Juli 1952	8.0	(0.00)	(0)

Stellt man die Resultate einander gegenüber, so findet sich in den heissen Jahrszeiten eine beträchtliche Verminderung der Ornithursäure bildung im Hühnerorganismus. Dagegen beträgt die Menge der

Ornithursäure im Winter über 10 fache der Sommerzeiten. Von Februar bis März ist die Ornithursäure-paarung am stärksten.

Brucinsalz der Ornithursäure

Obschon die erhaltene Ornithursäure optisch aktiv ist, schien es uns doch nicht überflüssig, ihr Verhalten gegen die optisch-aktiven Basen zu prüfen. Das Brucin lieferte kristallisiertes Salz. Man löst 5.2 g Brucin (Merck) in 150 ml. Alkohol und dazu versetzt man 5 g. Ornithursäure, die aus Hühnerexkrementen isoliert wurden. Wenn man die alkoholische lösung unter vermindertem Druck bis 80 ml. eingeengt und im Eisschrank stehen lässt, so scheidet sich aus der dicken Flüssigkeit im Laufe von einigen Stunden eine weise schuppenartige Kristallmasse ab. Ihre Menge beträgt 7.9 g. Aus der Mutterlauge gewinnt man noch 1.4 g. Die Substanz schmilzt unscharf bei 134–144:

Spaltung des Brucinsalzes der Ornithursäure

9 g. Brucinsalz der Ornithursäure werden in 150 ml. Wasser suspendiert und unter starker Kühlung (−6° bis −12°) mit 150 ml. *N*-NaOH versetzt. Das abgeschiedene Brucin wird nach 1 stündigen Stehenlassen bei −6° möglichst scharf abgenutscht, zuerst mit Chloroform und dann mit Aether ausgeschüttelt. Die wässerige Lösung wird jetzt mit 150 ml. *N*- HCl angesäuert und unter stark vermindertem Druck eingeengt. Wird die Lösung im Eisschrank abgekühlt, so beiginnt die Kristallisation. Aus heissem Alkohol umkristallisiert, schmilzt sie bei 184°. Gesamtausbeute 3.7 g.

Spaltung der Ornithursaure durch HBr

Zur Hydrolyse der Ornithursäure ist Bromwasserstoffsäure zu empfehlen, da sie leichter als Salzsäure von der freien Aminosäure getrennt werden kann. 3.5 g. Ornithursäure, die aus Hühnerexkrementen isoliert wurden, werden mit 50 ml. 25 proz Bromwasserstoffsäure 4 Stunden am Rückflusskühler gekocht. Nach dem Abfiltrieren der ausgeschiedenen Benzoesäure wird did Flüssigkeit mehrmals ausgeäthert und unter vermindertem Druck zum Sirup eingedampft. Der saurer reagierende Sirup wird mit Ammoniak genau neutralisiert und in Alkohol gelöst. Wenn man dazu Aether versetzt, so fällt Ornithinmonobromhydrat sofort kristallinisch aus. Weisse schuppenartige Kristallchen werden mit Aether und Alkohol-Aether Lösung (3 : 1) auswaschen. Sie schmelzen bei 225°. Ausbeute 0.8 g.

Ber. für $C_5H_{12}N_2O_2 \cdot HBr$ C 28.44% H 6.18%
Gef. 28.16 6.10

Aus dem hier erhaltenen Ornithin-mono-bromhydrat konnte man leicht Orithursäure synthetisieren. Aus 0.17 g. Ornithin-mono-bromhydrat wurde 0.11 g. Ornithursäure erhalten.

Die Elementaranalysen wurden in dem Organisch-chemischen Institut der Universität zu Kyoto ausgeführt. Wir sagen dafür Herren Prof. R. Nozu und Prof. R. Goto besten Dank.

LITERRATURE

(*1*) Crowdle J. H., u. Sherwin C. P., *J. biol. Chem.*, **55**, 365 (192
(*2*) Jaffé, M., *Ber. dtsch. chem. Ges.*, **10**, 1925 (1877)
(*3*) Sendju, Y., *J. Biochem.*, **7**, 273 (1927)
(*4*) Takahashi, M., *Z. physiol. Chem.*, **178**, 294 (1928)
(*5*) Thomas, K., *Col. Physiol.*, **28**, 769 (1914)
(*6*) Totani, G., *Z. physiol. Chem.*, **68**, 75 (1910)